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[2]Passed away on June 11, 2007
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EDITORIAL

Intrahepatic cholestasis of pregnancy

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Abstract

Intrahepatic cholestasis of pregnancy (ICP) is a pregnancy-specific liver disorder characterized by maternal pruritus in the third trimester, raised serum bile acids and increased rates of adverse fetal outcomes. The etiology of ICP is complex and not fully understood, but it is likely to result from the cholestatic effects of reproductive hormones and their metabolites in genetically susceptible women. Equally unclear are the mechanisms by which the fetal complications occur. This article reviews the epidemiology, clinical features, diagnosis, etiology and management of ICP.

Key words: Cholestasis; Pregnancy; Pruritus; Bile acid

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INTRODUCTION

Intrahepatic cholestasis of pregnancy (ICP), which is also known as obstetric cholestasis, is a liver disease of pregnancy associated with raised serum bile acids and increased rates of adverse fetal outcomes.

ICP was originally described in 1883 by Ahlfeld as recurrent jaundice in pregnancy that resolved following delivery. Pruritus was not mentioned in this report, but in subsequent case reports published in the 1950s, severe pruritus with or without jaundice was reported in conjunction with the condition, in addition to complete resolution following delivery and high recurrence rates in subsequent pregnancies[1,2].

Over the years, ICP has also been described as jaundice in pregnancy, recurrent jaundice in pregnancy, idiopathic jaundice of pregnancy, obstetric hepatosis, hepatosis gestationalis or obstetric cholestasis.

Most authors now agree that ICP should be defined as pruritus with onset in pregnancy, which is associated with abnormal liver function in the absence of other liver disease and which resolves following delivery.

EPIDEMIOLOGY

The incidence of ICP varies widely with geographical location and ethnicity (Table 1)[3-27]. It is most common in South America, particularly in Chile, where early reports described an overall incidence of 10%, with higher rates seen in women of Araucanian Indian descent[6]. More recently, this has fallen to approximately 1.5%-4%[28]. The reasons for this decline are unclear but do not appear to reflect changing diagnostic criteria which have become more inclusive in recent studies; while early reports commonly used jaundice to diagnose ICP, more recently, any abnormality in liver function has been used. Instead, it has been proposed that the decline is due to changes in environmental factors, which will be discussed in more detail later in this article. The incidence of ICP is lower in Europe (approximately 1%) and has been stable for many years.

ICP is more common in the winter months in Finland, Sweden, Chile and Portugal[23,24]. A higher incidence is seen in twin pregnancies (20%-22%)[10,18] and following *in vitro* fertilization treatment (2.7% *vs* 0.7%)[29]. One study has suggested that it is more common in women over the age of 35 years[11]. There is a higher incidence of gallstones in both affected women and their families[30,31]. Hepatitis C seropositivity has been reported to be a risk factor for ICP, and may be associated with early onset of the condition[15,32]. It has also been suggested that women with ICP have more severe and prolonged emesis, and higher rates of drug sensitivities[33].

Table 1 The reported incidence of ICP in different countries and ethnic groups

Country	Prevalence (%)	Year of study	Diagnostic criteria	References
Australia	0.2	1964-1966	P, J, LFT, PR[1]	[5]
Australia	1.5	1968-1970	P, J, LFT, R	[8]
Australia	0.2	1975-1984	P, J, LFT, PR; SBA (from 1982)	[3]
Bolivia	9.2	1976	P, J, B, LD	[7]
Aimaras	13.8			
Quechas	4.3			
Caucasians	7.8			
Mixed Indian	7.3			
Canada	0.07	1963-1976	J, P, LFT, LD, R	[4]
Chile		1974-1975	P, J, B, LD	[6]
Aimaras	11.8			
Araucanian	27.6			
Caucasian	15.1			
Chile	4.7	NA	P, SBA (10 μmol/L), B, LFT	[10]
Chile	6.5	1988-1990	P, LD[2]	[18]
China	0.32	1981-1983	J, LFT, B, SBA, LD, R[3]	[12]
Chongqing				
China	0.05	2003-2005	LFT, SBA (11 μmol/L), B, LD	[14]
Hong Kong				
Finland	1.1	1971-1972	P, LFT	[13]
Finland	0.54	1990-1996	P, LFT, SBA (8 μmol/L), LD	[11]
Finland	0.54	1994-1998	P, LFT, SBA (8 μmol/L), LD	[9]
France	0.2	1953-1961	P, J, LFT, PR, R	[16]
France	0.53	1988-1989	LFT, B, SBA (6 μmol/L)	[19]
India	0.08	2002-2004	P, LD, LFT	[17]
Italy	0.96	1996-1999	P, LFT, SBA[4]	[15]
Italy	1	1989-1997	P, PR, LFT ± SBA	[20]
Poland	1.5	NA	P, LFT, B, LD	[21]
Portugal	1	NA	P, SBA, LFT, B, LD[5]	[24]
Sweden	1.5	1971-1974	P, LFT, LD	[23]
	1	1980-1982		
Sweden	1.5	1999-2002	P, SBA (10 μmol/L), LFT, LD	[25]
USA	0.32	1997-1999	P, SBA or LFT, PR[6]	[26]
USA	5.6	1997-1998	P, SBA (20 μmol/L)	[27]
Latina				
UK	0.7	1995-1997	P, SBA (14 μmol/L), LFT, LD	[22]
Caucasian	0.62			
Indian	1.24			
Pakistani	1.46			

P: Pruritus; J: Jaundice; LFT: Raised AST and/or ALT; SBA: Raised serum bile acids [upper limit of normal defined as in the study (μmol/L)]; B: Raised bilirubin; PR: Postnatal resolution; LD: Other liver diseases excluded; R: Recurrence in subsequent pregnancy; NA: Information not available. [1]Absence of parenchymal necrosis on liver biopsy; [2]Absence of fever or malaise, LFT's only performed if jaundiced, dark urine or doubt over diagnosis; [3]Absence of hepatomegaly; [4]Absence of gallstones; [5]Increased cholic acid percentage; [6]Absence of other hepatic disease associated with pregnancy.

CLINICAL FEATURES

Maternal disease

The most common presenting symptom of ICP is pruritus that usually presents in the third trimester. This becomes progressively more severe as the pregnancy advances and typically resolves within 48 h of delivery. Pruritus is defined as an unpleasant sensation that evokes the desire to scratch. It most frequently affects the palms of the hands and soles of the feet but it can be generalized or affect other areas of the body. There are no associated dermatological features other than excoriation marks, which may be severe. Many women report that their pruritus worsens at night and may become so extreme that it causes insomnia.

Approximately 80% of affected women present after 30 wk of gestation[30,34], but ICP has been reported as early as 8 wk[23].

The relationship between onset of pruritus and development of deranged liver function is not clear. It has been reported that itch may be present either prior to or after abnormal liver function is detected[35], and this may reflect the heterogeneous nature of the condition.

Clinical jaundice is rare, affecting approximately 10%-15% of pregnant women with ICP, and if it does occur, it tends to be mild with bilirubin levels rarely exceeding 100 μmol/L. Unlike the pruritus, it does not typically deteriorate with advancing gestation[36].

Constitutional symptoms of cholestasis may also be present, including anorexia, malaise and abdominal pain. Pale stools and dark urine have been reported and steatorrhea may occur[37]. Theoretically steatorrhea is associated with an increased risk of post-partum haemorrhage as a result of malabsorption of vitamin K, although there are only a small number of reports of this complication in the literature[38]. Steatorrhea may respond to treatment with pancreatic enzymes.

There have been some reports of the co-existence of ICP with other pregnancy-related disorders including pre-eclampsia[14,39-41], acute fatty liver of pregnancy[30,42], and gestational diabetes[39]. This reflects the etiological heterogeneity of the condition and thus it is important to exclude other causes of hepatic impairment in women who present with cholestasis in pregnancy.

ICP is not typically associated with ongoing hepatic impairment after pregnancy and the biochemical abnormalities normally resolve within 2-8 wk of delivery. There are a few case reports of a more prolonged course with biochemical abnormalities lasting up to 34, 45 and 82 wk postpartum[43,44]. In women with continued liver dysfunction it is important to exclude alternative underlying diagnoses. In the majority of women, ICP recurs in subsequent pregnancies, but disease severity cannot be predicted by the course in previous pregnancies.

Fetal disease

There is considerable debate in the literature about the extent of the ICP-associated fetal risk. There are consistent reports of adverse fetal outcomes in association with the condition[45,46], although most studies are not sufficiently large to allow accurate quantification of the frequency of the complications. Many studies have tried to correlate maternal serum biochemistry with fetal outcomes and one series reported higher

Table 2 The incidences of adverse fetal outcomes reported in the literature

Study period	Number of cases (controls)	Preterm delivery (< 37 wk)	Abnormal CTG (timing)	Apgar score ≤ 7 (min)	Meconium staining of amniotic fluid	PPH (> 500 mL)	References
1951-1983	100 (156)	38% 38% U	-	-	-	7%	[12]
1963-1976	42 (42)	39% 39% S	-	-	-	19%	[4]
1965-1974	56	36% 36% S	14% (NS) 8 B	-	27% NS	9%	[38]
1971-1972	116 (116)	-	19% (labour) 3% LD 16% B or T	7% (1) 3% (5)	28% 7% < 37 wk 21% > 37 wk	-	[13]
1971-1974	100 (100)	-	-	10% (1) 8% (5)	12% NS	-	[23]
1975-1984	83	44% 44% S	-	-	45% 10% < 37 wk 35% > 37 wk	-	[46]
1979-1981	18	-	-	-	58.30% NS	22%	[39]
1980–1981	117	-	14% (antepartum) 4% (labour)	10% (NS)	16.20% NS	-	[48]
1988-1990	320 (320)	19% 12% S 7% I	12.8% (NS)	8% (1) 2% (5)	38% 13% < 37 wk 25% > 37 wk	-	[18]
1988-1995	79 (79[2])	14% 14% S	2% (NS) 2% B	None	44% NS	-	[57]
1989-1995	50	60% 60% U	-	-	-	2%	[56]
1989-1997	206	27% 4% S 23% I	-	1% (5)	21% 5% < 37 wk 16% > 37 wk	-	[20]
1990-1996	91 (16, 818)	14% 14% U	20% (labour)	8% (1) 2% (5)	15% NS	-	[11]
1999-2001	70	17% 6% S 11% I	-	-	14% 4% < 37 wk 10% > 37 wk	17%	[30]
1999-2002	690 (44, 792)	12% 12% U	7%[3]	7%[3]	25%	-	[25]
1999-2003[1]	352	38% 16% S 22% I	-	-	-	-	[31]
2000-2007	122	4% 4% S	-	8% (1) 2% (5)	13%	7%	[49]
2003-2005	8	50% 50% U	-	-	62% NS	-	[14]

S: Spontaneous preterm labour; I: Iatrogenic preterm labour; U: Unspecified preterm labour; NS: Not specified; B: Bradycardia; T: Tachycardia; LD: Late decelerations; PPH: Post-partum hemorrhage; -: Not reported. [1]Cases were recruited between 1999 and 2003, but the affected pregnancies had occurred from 1986; [2]Controls in this study had a history of stillbirth in a previous pregnancy; [3]Glantz *et al*[25] report findings of asphyxial events (defined as operative delivery due to abnormal CTG, post-partum umbilical artery pH < 7.05 or Apgar score < 7 at 5 min).

rates of fetal complications in women with jaundice compared to those with pruritus alone[38]. Bile acids have been repeatedly implicated in the etiology of the fetal disease, and the sensitivity of bile acids as a predictive marker of fetal risk has been examined in several studies involving small numbers of cases[18,39,47,48]. A recent, more definitive study from Sweden reported that there was a 1%-2% increase in risk of spontaneous preterm labour, asphyxial events (defined as operative delivery due to asphyxia, Apgar score < 7 at 5 min or arterial cord pH < 7.05) or meconium staining of the amniotic fluid and/or placenta and membranes for every additional μmol/L of maternal serum bile acids[25]. This study also reported no increase in adverse outcomes if the maternal fasting serum bile acids were below 40 μmol/L, leading the authors to suggest there is no increased risk to the fetus with mild ICP according to this definition. This result was generally consistent with the findings of other smaller studies in Finnish and American Latina populations[47-49] (Figure 1), although the magnitude of the effect varied in different studies, possibly relating to variations in management strategy, maternal ethnicity and study design. The reported incidence of each adverse fetal outcome is shown in Table 2.

Meconium staining of the amniotic fluid: The incidence of meconium staining of amniotic fluid (MSAF) in normal term pregnancies is approximately 15% and is considered to be a sign of fetal distress. In ICP, MSAF has been reported in 16%-58%[38,39] of all

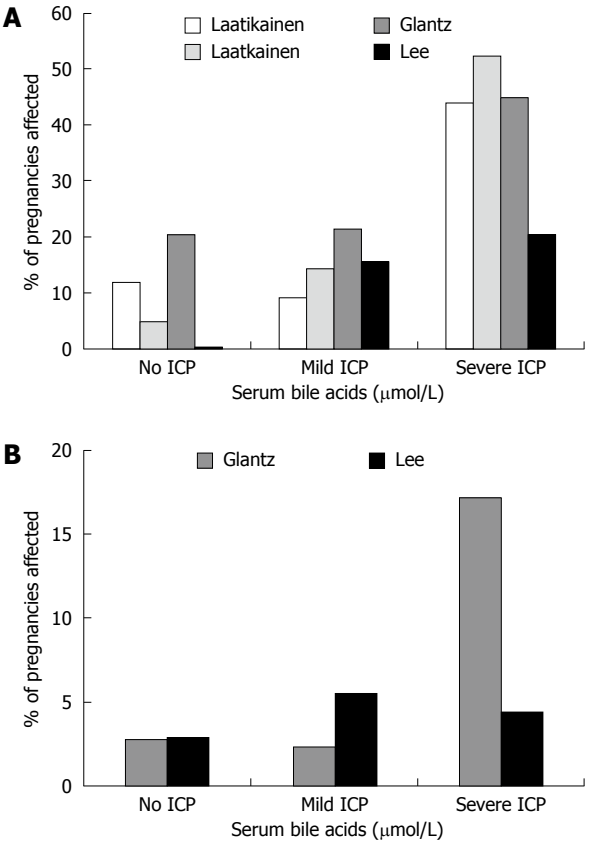

Figure 1 Graphs showing the incidence of meconium staining of the amniotic fluid (A) and preterm labour (B) in studies of the relationship between the maternal serum bile acid level and adverse fetal outcomes[25,47-49]. The total maternal serum bile acid level was used in all studies except Laatikainen *et al*[47] (represented by the white bar), where cholic acid only was used (normal range < 5 μmol/L). Serum bile acid level has been categorized as no ICP (< 10 μmol/L), mild ICP (10-40 μmol/L) or severe ICP (> 40 μmol/L) by the authors. One study[25] used fasting maternal bile acids and compared fetal outcomes with the single highest bile acid reading available. The other studies did not specify whether the mothers were fasted. One study[48] compared fetal outcomes to the serum bile acid level from the week before delivery, and the other studies did not specify which serum bile acid level was used.

cases and up to 100% of cases affected by intrauterine death (IUD)[45]. The group in which the incidence of MSAF is particularly striking is women who had amnioscopy or amniocentesis at approximately 37 wk of gestation, as the rates are significantly higher than in controls at this time[20]. The frequency of MSAF is greater in pregnancies with higher reported levels of maternal serum bile acids[25,47-49] (Figure 1).

Cardiotocography (CTG) abnormalities: Both ante- and intrapartum CTG abnormalities have been reported in association with ICP, including reduced fetal heart rate variability, tachycardia and bradycardia (< 100 bpm)[13,38,48,50]. More recently, a case report has described fetal tachyarrhythmia (220-230 bpm) leading to atrial flutter during labour at 37 wk gestation[51].

Preterm labour: There is an increased risk of spontaneous preterm labour, which has been seen in as many as 60% of deliveries in some studies[4], but most studies report rates of 30%-40% in ICP cases without active management. Reid *et al*[38] found an overall incidence of 36%, but interestingly this rose to 48% in women with raised bilirubin. Two studies have related the maternal serum bile acid level to the rate of spontaneous preterm delivery[25,49]. The rate of this complication was significantly higher in ICP pregnancies with maternal fasting serum bile acids > 40 μmol/L in the larger study of Swedish ICP cases[25]. However it was not higher in pregnancies with mildly raised (< 20 μmol/L) or more markedly raised maternal serum bile acids in a study of American Latina cases[49].

In more recent studies, the majority of preterm deliveries are iatrogenic (Table 2), which reflects the relatively frequent practice of electively delivering ICP cases at around 37 wk, with the aim of reducing the risk of fetal complications. There have been concerns raised over whether this in itself carries an increased risk of neonatal morbidity for the fetus. Studies have shown that there is an increased risk of respiratory distress syndrome (RDS) with either induction of labour or elective cesarean section at this stage of gestation[52]. It should be noted that the risk of neonatal respiratory distress is considerably higher with elective cesarean section, and it should be borne in mind that labour is induced in the majority of women with ICP. Also, there are some data to suggest that neonatal respiratory distress following ICP may be a consequence of the disease process. A recent series reported unexpected respiratory distress in association with maternal cholestasis in three infants delivered between 36 and 37 wk gestation with good indices of lung maturity in the amniotic fluid and negative blood, urine and cerebrospinal fluid cultures[53]. The authors therefore proposed that the development of RDS was as a direct consequence of ICP. In follow-up studies, RDS was found to affect 28.6% of newborns from cholestatic pregnancies and high levels of bile acids were found in the bronchoalveolar fluid of 10 infants with RDS[54,55].

Sudden IUD: Older studies using biochemical abnormalities to diagnose ICP have reported a perinatal mortality rate of 10%-15%[13,38]. This has been reduced to 3.5% or less in more recent studies employing policies of active management[11,13,18,20,25,30,39,46,48,56]. The term active management may encompass many different clinical practices, including increased fetal monitoring, frequent biochemical testing, pharmacotherapy with ursodeoxycholic acid (UDCA) or delivery at 37-38 wk gestation. These management protocols are based on evidence showing that stillbirths in ICP tend to cluster around 37-39 wk (Figure 2)[13,18,23,25,31,38,45-48,56-58]. However, there have been reports of stillbirths at less than 37 wk; in one series a fetus died at 32 wk[45] and in another one, twin fetuses died at 31 wk[31].

The risk of adverse fetal outcomes is thought to relate to the maternal serum bile acid level, and a recent study has shown that there is a 1%-2% increased risk for every μmol/L of bile acid above 40 μmol/L[25]. It is therefore likely that the risk of IUD is higher in

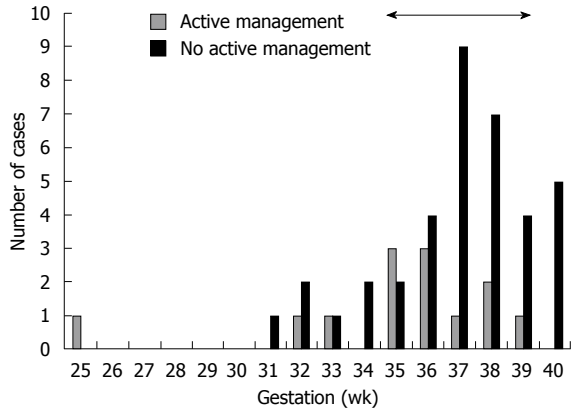

Figure 2 Graph showing the timing of IUD associated with ICP[13,18,23,25,31,38,45,47,48,56-58]. The arrow represents six additional cases of IUD from two series with no active management reported as a range of gestational ages at the time of fetal death[45,46].

ICP pregnancies with more severe hypercholanemia. However, the same study reported a stillbirth with maternal serum bile acid levels of 27 μmol/L and there are additional case reports of stillbirths at 39 wk with bile acids of 15 and 21 μmol/L[48,58]. It is not clear how close to the fetal death these blood specimens were taken, and maternal serum bile acid level is high in the majority of IUDs reported in the literature[25,47], but these observations illustrate the difficulty encountered by clinicians in using biochemical measurements to dictate delivery strategies for ICP cases.

Other findings: Several studies have shown that there is no increase in the number of small for gestational age infants born to women with ICP[18,59]. However, lower mean birth weight has been noted in three studies[4,11,38], although this does not appear to be due to intrauterine growth restriction. One study reported an increased placental/fetal mass, i.e. larger placentas in ICP[11].

INVESTIGATIONS

The diagnosis of ICP is one of exclusion and alternative causes of hepatic impairment or pruritus should be considered before the diagnosis is made.

Liver function tests (LFTs)

Liver function in normal pregnancy: Liver function does not change in normal pregnancy, although it is recommended that adjusted upper limits of normal are used. The upper limit of the normal reference range for serum alanine transaminase (ALT) and aspartate transaminase (AST) should be reduced by 20%[60] and the γ-glutamyl transpeptidase (GGT) level is reduced by a similar amount in later pregnancy[61]. Total and free bilirubin is also lower during all three trimesters, and conjugated bilirubin is lower in the second and third trimesters[61].

LFTs in ICP: The transaminase enzymes are located within hepatocytes and raised serum levels are thus indicative of hepatocellular damage. In ICP, ALT and AST may rise before or after serum bile acids[39,62]. Of the two, ALT is thought to be a more sensitive marker of ICP; there is a 2-10-fold increase in serum levels that is generally more marked than the rise in AST[3,47,62].

Bilirubin is normal in the majority of ICP cases and is of limited value in diagnosis or follow up. If raised, it tends to be a conjugated hyperbilirubinemia[62].

GGT has been shown to be raised in some studies[24,56,63] but is more commonly normal[47]. It has been proposed that elevations in GGT are associated with a greater impairment in other LFTs[63], and that they can provide insights into the genetic etiology of the condition.

Alkaline phosphatase (ALP) levels may rise in ICP but production of large amounts of the placental isoform render this biochemical marker of limited diagnostic value.

Glutathione *S*-transferase alpha (GSTA) is a phase Ⅱ detoxification enzyme that is rapidly released into the circulation following acute hepatic damage. It is reported to be a more sensitive and specific marker of hepatic integrity than standard LFTs[64-66]. A longitudinal study comparing serum levels from ICP, control and pruritus gravidarum cases demonstrated higher GSTA in ICP at all gestations from 24 wk to term[67]. A recent study confirmed that GSTA levels are elevated in ICP cases[68], and the authors of both studies proposed that it may be a useful tool for early diagnosis of the condition.

Bile acids: The primary bile acids cholic acid (CA) and chenodeoxycholic acid (CDCA) are the end products of hepatic cholesterol metabolism and represent the major route for excretion of cholesterol. Following synthesis, CA and CDCA are conjugated with taurine or glycine (in a ratio of approximately 1:3) before export across the canalicular membrane to enter the bile. In the terminal ileum and colon, CA and CDCA undergo bacterial modification, mainly deconjugation and 7α-dehydroxylation, to form the secondary bile acids deoxycholic acid (DCA) and lithocholic acid (LCA), respectively. UDCA is a tertiary bile acid as it results from bacterial modification followed by hepatic metabolism. It is normally detectable in trace amounts in normal serum. Bile acids are reabsorbed in the terminal ileum and enter the portal vein for transport back to the liver. The enterohepatic circulation is highly efficient and 95% of all bile acids are reabsorbed. A more detailed account of bile acid synthesis, canalicular secretion, sinusoidal uptake and homeostasis is given in a number of recent reviews[69,70].

Bile acids in normal pregnancy: In healthy pregnancy, there is a minimal rise in total serum bile acids as gestation advances[71,72]. Studies in which individual bile acid levels have been measured show that there is no change in DCA but CDCA doubles by term[73]. The data concerning CA are not so conclusive, with some studies reporting a significant increase in the third

trimester compared to the first[59,72], and others showing no change[73]. However, in all studies the level stayed well within the normal range (< 1.5 μmol/L). Perhaps a more informative measure is the ratio of the two primary bile acids (CA:CDCA), which is reported to be between 0.68 and 1.9 at term (38-40 wk)[72,73].

The reference range used for total serum bile acids in pregnancy varies, but most authors accept an upper limit of normal of between 10 and 14 μmol/L.

The phenomenon of asymptomatic hypercholanemia of pregnancy (AHP) has recently been reported. AHP is defined as raised serum bile acids in pregnancy in the absence of symptoms and other biochemical markers of ICP[74]. Specifically, they describe a change in the profiles of serum bile acids in women with AHP with increased CA and relatively unchanged CDCA levels. AHP is reported to affect approximately 10% of the pregnant population, and 2%-3% of women with AHP during the second trimester subsequently develop ICP.

More recently, Castaño *et al*[75] reported that AHP affects 40% of pregnant women in Argentina and that the pregnancy outcome is similar to that of normal pregnancies, suggesting that mild hypercholanemia may fall within the biochemical spectrum of normal pregnancy.

Bile acids in ICP: Serum bile acid measurement is now considered to be the most suitable biochemical marker for both the diagnosis and monitoring of ICP[76], with the cholic acid level[59] or the CA:CDCA ratio proposed as being the most sensitive indicator for the early diagnosis of the condition[24,62]. Levels of the secondary bile acid DCA also rise[73], although to a lesser extent, and indicate impairment of the enterohepatic circulation.

There are reports in the literature of elevations in serum bile acids as high as 100 times the upper limit of normal[73,77].

In addition, maternal cholestasis results in elevated levels of bile acids in the fetal circulation and a reversal in the normal feto-maternal transplacental bile acid gradient[39,78,79].

There is as yet no consensus on whether a rise in serum bile acids precedes the onset of symptoms. There are reports of women with elevated serum bile acids prior to the onset of symptoms or the appearance of other biochemical abnormalities[62]. Equally, there is no agreement on whether serum bile acids should be measured in the fasted or post-prandial state. In ICP, consumption of a standard test meal causes a more dramatic and prolonged post-prandial rise in serum bile acids, particularly CA, than in control women, suggesting that the use of a standard test meal may help to distinguish mild forms of the disease from normal[80,81]. However, this approach would be costly and time-consuming if used in routine obstetric practice.

The biliary bile acid profile is also altered in ICP. Although CA remains the predominant bile acid, the proportion is greatly increased, with a consequent reduction in the proportion of CDCA. DCA is markedly diminished, suggesting a significant impairment of the enterohepatic circulation in ICP[82].

Other serum biochemistry

Lipids: Cross-sectional studies have reported deranged lipid profiles in association with ICP[34,83,84], and in a prospective longitudinal study LDL cholesterol, apolipoprotein B-100 and total cholesterol concentrations were found to be elevated[85]. However, it should be noted that this study was performed in a group of patients who were not fasted at the time of sample collection.

Glucose: One study has shown that ICP is associated with impaired glucose tolerance. Although there was no difference in fasting glucose levels between cases and controls, both the 2 h post-prandial glucose and oral glucose tolerance tests were higher in ICP[21].

Clotting: One study reported a prolonged prothrombin time in 20% of patients[12]. However this is not consistent with the author's experience. In 65 ICP cases with raised serum bile acids managed in our hospital from 1996-2003, there were none with an abnormal clotting profile.

Other biochemical markers investigated in ICP are summarized in Table 3[86-100].

Urine

Analysis of the urine from women with ICP shows an increased excretion of total bile acids, with a 10- to 100-fold increase in CA and CDCA, but decreased excretion of DCA and LCA. The reduced excretion of the secondary bile acids supports the hypothesis that a canalicular defect is a primary feature of ICP, and is consistent with an impaired enterohepatic circulation. The bile acid profile shows a shift from glycine to taurine conjugation and an increased proportion of sulfated species[101]. In a longitudinal study of two women, the first detectable change in urinary bile acids was the appearance of tetrahydroxylated species, which are known products of CA metabolism. This change occurred before the rise in total bile acid excretion[102].

Liver/gallbladder ultrasound scan

Gallstones are reported in 13% of women with ICP[31]. While it is likely that affected women have increased susceptibility to cholelithiasis, there have been no robust studies of ICP cases and controls, and pregnancy itself is also associated with an increased incidence of asymptomatic gallstones[103,104]. First-degree relatives of affected women also have higher rates of cholelithiasis (26% of relatives of 227 ICP cases compared to 9% of 234 controls, $P < 0.001$, χ^2) (unpublished data, C. Williamson).

In ICP, the intrahepatic bile ducts appear normal, but the fasting and ejection volumes of the gallbladder are greater, possibly predisposing these women to the

Table 3 Additional biochemical markers that have been investigated in relation to ICP

Number of cases (controls)	Gestation	Parameter investigated	Main findings	References
76	NR	Serum human placental lactogen and AFP	↑ hPL, no difference in AFP	[88]
68	37-38/40	Pregnancy specific β-1-glycoprotein	↓ Levels	[89]
76 (150)	T3	Prolactin	↑ Prolactin from 33 wk	[94]
10 (288)	T3	Placental protein 10	↓ Placental protein 10 in ICP-negatively correlated with AST and SBA	[98]
NR	T3 + PN	Serum copper and zinc	↑ Copper in ICP No difference in zinc	[91]
NR	T3	Serum 25(OH)D, 24, 25(OH)D, 1, 25(OH)D, total protein, calcium, phosphorus, magnesium and alkaline phosphatase	25(OH)D initially higher in patients than controls, but decreased by delivery No change in any other parameter	[92]
12 (12)	T3	Serum selenium and glutathione peroxidase	↓ Selenium in ICP ↓ Glutathione peroxidase activity	[90]
33 (5680)	T2	Serum AFP and hCG	No differences	[87]
26 (13)	NR	Thyroid hormones	↓ T3	[93]
21 (98)	T3	Serum selenium, zinc and copper	↓ Selenium in ICP ↑ (Double) copper in ICP No difference in zinc	[96]
72 (30)	T3	Renal function tests: uric acid, urea, potassium, sodium, creatinine	↑ Uric acid and creatinine	[97]
22 (21)	NR	Maternal-fetal mixed lymphocyte reaction	↓ Transformation rate of lymphocytes	[86]
24 (1148)	T3	Serum AFP and β-hCG	No differences	[95]
30 (30)	T3	Serum neopterin and soluble interleukin 2 receptor (sIL-2R)	↑ Neopterin and sIL-2R	[99]
58 (42)	T3	Alpha-hydroxybutyrate dehydrogenase (α-HBDH) activity	↑ α-HBDH	[100]

NR: Not reported; T3: Third trimester; T2: Second trimester; PN: Postnatal.

formation of gallstones[105-107]. However, ICP has been described in women with previous cholecystectomy[108], suggesting that the presence of gallstones is not causative of ICP.

Liver biopsy

Several studies have reported that there is normal liver structure with no evidence of liver cell damage and only mildly dilated bile ducts, bile stasis in canaliculi, bile plugs and mild portal tract inflammation in liver biopsies from women with ICP[5,109]. Electron microscopy findings show generally well-preserved architecture with dilated bile capillaries, distorted microvilli and granular deposits (bile thrombi)[5,109].

ETIOLOGY OF MATERNAL DISEASE

The etiology of ICP is complex and not fully understood. Evidence from ICP pedigrees suggests that there is a genetic component to the disease[110], and the relative risk for parous sisters of affected women is 12[9,111]. Insights into the genetic etiology come from studies of the familial cholestasis syndromes progressive familial cholestasis (PFIC) and benign recurrent cholestasis (BRIC). These autosomal recessive syndromes are caused by homozygous mutations in the genes encoding biliary transport proteins, and case reports have described ICP in the heterozygous mothers of affected children.

Candidate genes

The most extensively studied candidate gene in ICP is *ABCB4*, which encodes the multidrug resistance protein 3, a floppase that transports phosphatidylcholine from the inner to the outer leaflet of the hepatocyte canalicular membrane[112-114]. Homozygous mutations result in a spectrum of phenotypes that include PFIC type 3[115] and cholelithiasis[116]. ICP-associated variants were first described in a case report published in 1999, in which the mother of a child with PFIC type 3 was found to have a heterozygous single nucleotide deletion (1712delT). She and five of her female relatives had a history of ICP[117]. However, a second study screening 57 Finnish cases for this mutation concluded that it does not play a significant role in the etiology of ICP in this population[118]. Subsequent to this, there have been 12 different genetic variants and four splicing mutations in *ABCB4* reported in ICP cases[119-126]. The first *ABCB4* mutations were described in patients with elevated serum GGT levels, a biochemical phenotype that is also found in PFIC type 3, and not in types 1 and 2. However, some recent studies have described *ABCB4* variants in ICP patients with normal GGT[120,126]. A recent study has also described an *ABCB4* haplotype which is associated with the "severe" phenotype of serum bile acids > 40 μmol/L[127].

Homozygous mutations in the familial intrahepatic cholestasis one gene (FIC1, *ATP8B1*) cause PFIC type 1 and BRIC. The function of the FIC1 protein is disputed, but it is hypothesized to be an aminophospholipid translocase which transports phosphatidylserine from the canaliculus into the hepatocyte, thus maintaining membrane asymmetry and the function of the other biliary transporters embedded within the membrane. Variation in *FIC1* occurs in a small number of ICP cases, although the functional consequences are currently not known[128,129].

Genetic variation in the bile salt export pump (BSEP), encoded by *ABCB11* has also been reported in ICP. BSEP is located exclusively in the hepatocyte canalicular membrane and is the primary export pump for bile acids. Homozygous mutations in *ABCB11* are associated with PFIC type 2. Two studies investigated the role of *ABCB11* variation in Finnish ICP cases. One study reported that single nucleotide polymorphisms in exons 28 and 19 were susceptibility loci for ICP[130]. However, a subsequent study in a larger number of cases with a more diverse ethnic background failed to confirm these findings, suggesting that ICP is a genetically heterogeneous disease[131]. Further evidence for genetic heterogeneity was provided by a study of 16 individuals from two affected Finnish families. Segregation of haplotypes and multipoint linkage analysis of microsatellite markers in *ABCB11*, *ABCB4* and *ATP8B1* excluded genetic variation in these genes from playing a role in the etiology of ICP[132]. Other BSEP variants reported to be associated with ICP include N591S and the V444A polymorphism[123,126,133]. The latter of these variants is particularly interesting as it is also reported to be a susceptibility factor for estrogen-induced cholestasis[133]. A recent UK study demonstrated that two common PFIC2-associated mutations (E279G and D482G) and N591S are present as heterozygous variants in a small proportion of ICP cases. This study of 491 Caucasian ICP cases and 261 controls also demonstrated that the V444A allele is a significant risk locus for ICP in this population[134].

Genetic variation has also been reported in another biliary transporter, *ABCC2*, which encodes the multidrug resistance related protein 2 (MRP2). MRP2 exports organic anions including bilirubin into the bile. A study from South America has suggested that a polymorphism in exon 28 is associated with ICP[135].

The farnesoid X receptor encoded by *NR1H4* is the principal bile acid receptor and is responsible for the regulation of bile acid synthesis and transport within the liver. A recent study has described four heterozygous variants within FXR that are associated with ICP, three of which were shown to have functional effects[136].

A variety of other genetic loci have been investigated in ICP cases and the reader is referred to a recent review for full details of all studies to date[111].

Influence of hormones

Several studies provide evidence that reproductive hormones play a role in the etiology of ICP. The disease is more common in multiple than singleton pregnancies (20.9% *vs* 4.7% in one study)[10], and the symptoms may recur in a subgroup of affected women when taking the combined oral contraceptive pill[31]. In addition, most women present with symptoms of ICP in the third trimester when estrogen and progesterone levels are highest.

Most studies have focussed on estrogen rather than progesterone. In clinical studies, administration of ethinylestradiol to both men and women results in a decreased clearance of sulfobromophthalein, and this is further reduced in women with a personal history of ICP and their male relatives[137]. Furthermore, administration of the depot estrogen, ethinylestradiol propanolsulphonate to 20 healthy women significantly increased the total serum bile acid concentration, and in particular, the proportion of taurine conjugates[138]. *In vitro* studies have demonstrated that the cholestatic estrogen metabolite, 17-β-estradiol glucuronide, transinhibits the BSEP following excretion into the bile canaliculus by MRP2[139]. Supporting this hypothesis, administration of 17-β-estradiol glucuronide to rats causes endocytic internalization of BSEP in an MRP2 (Mrp2; Abcc2)-dependent manner[140]. Estrogen also impairs the expression and/or function of, BSEP and MRP2 in rodent studies[141-143].

However, progesterone may play an even greater role in the pathogenesis of ICP. Bacq *et al*[144] observed that administration of natural progestin to women with threatened preterm labour resulted in ICP in 11 of the 12 women treated. This finding was confirmed by two subsequent studies[56,145]. Although total progesterone does not rise in comparison to normal pregnancies, the profile of metabolites is considerably different. An excess of monosulfated and disulfated (in particular 3α and 5α) isomers has been reported in the serum and urine of women with ICP, and this may reflect impaired excretion of these metabolites at the canalicular membrane, or abnormal synthesis[146,147]. Studies of umbilical cord serum have also shown that disulfated progesterone metabolites are increased in the fetal compartment of affected pregnancies compared with normal pregnancies. Moreover, the level of steroid sulfates synthesized by the fetus, e.g. 16α-hydroxydehydroepiandrosterone sulfate are decreased, suggesting that maternal cholestasis is associated with impaired fetal steroid synthesis[148].

There are few *in vitro* studies that have investigated the mechanism of progesterone metabolite induced cholestasis. One study has shown that sulfated progesterone metabolites, but not progesterone itself, cause reduced bile flow in the rat[149]. The same authors demonstrated that these metabolites inhibit BSEP-mediated bile acid efflux in *Xenopus* oocytes.

Environmental factors

Selenium: Serum levels of selenium usually decrease with advancing gestation, but normal serum levels are maintained if dietary intake is adequate[96]. Dietary selenium intake is lower in Finland and Chile, and serum levels have been shown to be reduced in women with ICP compared to pregnant controls in both countries[90,96]. Glutathione peroxidase is a powerful antioxidant that is dependent on selenium. Estrogens and bile acids cause oxidative stress, and it has been proposed that reduced serum selenium levels may contribute to the etiology of ICP and may also provide an explanation for the geographic variation in the prevalence of the condition.

Seasonal variation: The incidence of ICP peaks in the winter months in Scandinavia and Chile, suggesting a possible association with an environmental trigger. Interestingly, serum selenium levels have also been reported to be significantly higher in the summer than winter[96].

Infection: There is an increased incidence of hepatitis C infection in women with ICP, and one study has reported that affected women develop cholestasis at earlier gestations[32]. There have also been reports of an increased incidence of urinary tract infection and pyelonephritis in the early stages of pregnancy than in controls[13].

Drug cholestasis: Johnston *et al*[4] reported higher rates of drug sensitivities among the ICP population, especially to antibiotics. There may be overlapping etiological factors that influence susceptibility to ICP and drug-induced cholestasis. For example, the V444A allele of BSEP that confers increased risk for ICP is also found more commonly in individuals with drug-induced cholestasis[150].

Leaky gut: Increased gut permeability as measured by the urinary lactulose/mannitol ratio (L/M) has been reported in a subgroup of ICP patients (five of the 20 women in the study), and the authors postulate that this may participate in the pathogenesis of the condition by enhancing the absorption of bacterial endotoxins[151]. However, levels of anti-lipopolysaccharide antibodies and pro-inflammatory cytokines [tumor necrosis factor-α, interleukin (IL)-1β, IL-6 and IL-10] were measured and no significant differences were shown, suggesting that this may not be the case. It should also be noted that the abnormal excretion of lactulose and mannitol persisted for up to 2 years in four of the five women identified, and thus it is possible that a leaky gut is a permanent abnormality in these women rather than a result of ICP.

Pruritus: The itch associated with ICP is often the most troubling symptom for affected women. It has been speculated that it is due to accumulation of bile acids in the interstitial fluid of the skin. However, serum bile acid levels do not correlate well with maternal symptoms and, while application of bile acids to blister bases or intradermal injection of bile acids results in pruritus[152,153], absolute concentrations of bile acids in the skin do not correlate well with the sensation of itch[154]. Furthermore, some studies have reported pruritus before the onset of biochemical abnormalities[35]. These findings suggest that an alternative compound acts as a pruritogen in this and possibly other forms of cholestatic liver disease. Candidates include reproductive hormone metabolites, and a recent study has reported that serum sulfated progesterone metabolites were reduced following treatment with UDCA, concurrently with a reduction of pruritus[155]. Interestingly, a 5-hydroxytrytamine 3 receptor agonist has been reported to rapidly reduce pruritus in both ICP and other liver diseases, raising the possibility that serotonin is involved in the etiology of pruritus[156,157].

ETIOLOGY OF FETAL COMPLICATIONS

The etiology of the fetal complications associated with ICP is poorly understood, but is thought to relate to an increased flux of bile acids into the fetal circulation, as indicated by elevated levels in amniotic fluid, cord serum and meconium. *In vitro* studies of isolated placental vesicles have shown that vectorial transfer of bile acids from fetus to mother is impaired in ICP, and that this is specifically the result of decreased efficiency of ATP-independent transport[158,159]. Taken together, these findings suggest that bile acids accumulate in the fetal compartment and thus are likely to exacerbate fetal risk. Furthermore, a recent study of fetal outcomes in ICP has shown that the risk of adverse fetal outcomes increases with increasing levels of maternal serum bile acids[25].

MSAF

Evidence for the involvement of bile acids in the etiology of MSAF comes from studies of fetal sheep infused with CA, in which 100% of the treated lambs were born with MSAF[160]. The mechanism by which bile acids cause this effect is not clear from this study: the lambs did not show any signs of fetal distress. However, bile acids are known to cause an increase in colonic motility[161,162] and this is a possible explanation. Alternatively, the bile acids may cause fetal distress and subsequent meconium passage.

CTG abnormalities

Individual neonatal rat cardiomyocytes treated with taurocholic acid show a decrease in the rate of contraction, which is reversible. Furthermore, cells in a network lose their ability to beat synchronously after the addition of taurocholic acid and have abnormal calcium dynamics, suggesting that elevated levels of bile acids in ICP may be responsible for the CTG abnormalities observed[163].

Spontaneous preterm labour

Rodent studies have shown that the non-pregnant rat myometrium displays a dose-dependent increase in contractility in response to CA[164], and sheep infused with this bile acid have an increased incidence of spontaneous preterm labour[160]. Furthermore, it has been suggested that the myometrium of ICP patients may be more responsive to the effects of oxytocin[165,166].

RDS

Bile acid aspiration or accumulation within the fetal circulation is thought to be responsible for the increased incidence of RDS seen in association with ICP. In animal models, bile acids have been shown to cause severe chemical pneumonitis and pulmonary edema[167,168].

Furthermore, intra-tracheal injection of bile acids in rabbits resulted in atelectasis, eosinophilic infiltration and formation of hyaline membrane, which could be reversed by the administration of surfactant[169]. It has therefore been hypothesized that elevated levels of bile acids in the fetal circulation cause a reversal of the action of phospholipase A2, thereby causing the degradation of phosphatidylcholine and a lack of surfactant[53]. Interestingly, administration of intra-tracheal surfactant to two of the infants reported in a recent series resulted in some improvement in their condition[53].

IUD

The mechanisms causing sudden IUD are poorly understood. At autopsy, the majority of the stillborn babies are of normal weight and have no signs of chronic utero-placental insufficiency, but do have signs of acute anoxia[38]. However, several studies have reported non-specific morphological changes in the placenta, including increased syncytial knot formation and villous edema, which are suggestive of hypoxic insults[47,170,171]. These findings are comparable to the morphological appearance of placentas from a rodent model of ICP, which is also associated with increased placental oxidative stress[172].

As previously discussed, there is MSAF in up to 100% of ICP-associated stillbirths. Studies of meconium from healthy pregnancies has shown that it penetrates deep into placental and umbilical cord tissue in less than 3 h[173], and can cause vasoconstriction of the placental and umbilical vessels. The mechanism is not known, but heat-inactivated meconium did not induce an effect in one study[174], suggesting that a peptide or prostaglandin produces the effect. In ICP, the meconium contains significantly elevated levels of bile acids (13.5 ± 5.1 μmol/g *vs* 2.0 ± 0.5 μmol/g)[175], and as bile acids are known to cause vasoconstriction of the placental chorionic vessels[176], it is possible that placental vasoconstriction is a mechanism that contributes to the risk of IUD in ICP.

MANAGEMENT OPTIONS

The aims of management are to reduce symptoms and biochemical abnormalities in the mother and to reduce the risk of fetal distress, preterm delivery and sudden fetal death.

Fetal monitoring

There are several case reports of normal CTG and/or normal fetal movements in the hours preceding fetal loss[18,48,177,178]. Thus, the general consensus is that these forms of fetal surveillance do not prevent IUD. However, they may be reassuring to women with ICP and the clinicians responsible for their care at the time they are performed. One study reported good fetal and neonatal outcomes with a policy of routine amnioscopy at 36 wk to assess amniotic fluid color in addition to standard monitoring for fetal wellbeing[20]. However, this approach may be considered overly invasive by many obstetricians.

Elective delivery

Some studies have reported good outcomes with a policy of induction of labour at 37 or 38 wk gestation[20,46]. Many clinicians in the UK have adopted this practice as the IUDs appear to cluster at later gestations (Figure 2). However there have been very few reports of the gestational week at which the IUD occurs, nor have there been any large prospective studies of whether drug treatment or early delivery prevents adverse fetal outcomes.

Drugs

UDCA: UDCA is a naturally occurring hydrophilic bile acid that constitutes < 3% of the physiological bile acid pool in humans. It has been used with positive effects in the management of primary biliary cirrhosis and other cholestatic disorders for several years, and is gaining popularity as a treatment for ICP. There is evidence that UDCA stimulates biliary secretion by post-transcriptional regulation of BSEP and the alternative exporters MRP4 and MRP3. In addition, it has antiapoptotic effects and has been shown to reduce the mitochondrial membrane permeability to ions and cytochrome c expression[179,180]. Finally, UDCA lowers serum levels of ethinyl-estradiol 17β-glucuronide, a major cholestatic metabolite of estrogen.

The first reported use of UDCA in ICP was by Palma *et al*[181] in 1992. In an uncontrolled series of eight cases, UDCA was prescribed at a dose of 1 g/d either continuously for 20 d or for two 20-d periods interrupted by a 14-d drug-free period. Both groups had a significant improvement in serum biochemistry and symptoms after 20 d treatment, but relapse was seen after the first week of the drug-free period in the latter group. Subsequently, UDCA was used to treat three patients with recurrent ICP, all of whom had rapid symptomatic and biochemical improvements with no adverse fetal outcomes[45]. This was followed by three small randomized, controlled trials (maximum of eight patients in each arm), the first of which showed that 20 d of UDCA treatment (600 mg/d) resulted in a significant reduction of pruritus and LFTs, including bile acids, compared to baseline[182]. One other study failed to show any reduction in pruritus compared with placebo, and the final one showed a reduction that did not reach statistical significance because of the small numbers of women treated[183,184]. In both studies, UDCA caused a significant reduction in serum transaminases and bilirubin compared to placebo. One study also showed a significant reduction in serum bile acids[183]. There have been several additional case series demonstrating that UDCA treatment results in clinical and biochemical improvement in ICP[26,185-188].

More recently, a randomized placebo-controlled trial comparing the efficacy of UDCA and dexamethasone therapy in ICP reported that UDCA, but not dexa-

methasone, significantly reduced ALT and bilirubin in all women treated. Furthermore, there was a significant reduction of pruritus and bile acids in women with serum bile acid levels exceeding 40 μmol/L at inclusion[189].

Studies examining the bile acid pool composition have shown that, in addition to a reduction in the serum bile acid concentration, treatment with UDCA results in a normalization of the CA:CDCA and glycine:taurine ratios[190], and a reduction in urinary excretion of sulfated progesterone metabolites, which the authors propose is associated with a concurrent reduction in pruritus[155].

There have been no reports of fetal morbidity or mortality resulting from UDCA treatment, although no study has had sufficiently large numbers to allow this to be fully evaluated. However, UDCA treatment has been shown to reduce the bile acid level in cord blood[187], amniotic fluid[187,191,192] and colostrum[193], and it reduced cord blood bilirubin levels in one study[194]. As previously discussed, the level of bile acids in meconium is considerably elevated in ICP, and this is not influenced by treatment with UDCA[175]. However, this may be because bile acids had already accumulated in the meconium prior to UDCA treatment. It is likely that, if UDCA reduces the maternal serum bile acid level, and thus placental transfer of bile acids, then there should be a corresponding reduction in the level in meconium from the time of treatment.

Finally, UDCA has been shown to correct the impaired bile acid transfer kinetics observed in ICP placentas[159] and to reverse the morphological changes seen in the placentas of a rodent model of ICP[195]. In addition, placental MRP2 protein and mRNA expression were significantly increased in patients treated with UDCA compared to controls[194]. UDCA also protects cardiomyocytes from bile acid-induced arrhythmias in an *in vitro* model[196].

There are very few side effects reported with UDCA treatment. At higher doses women may complain of gastrointestinal upset and diarrhea, but this is rare.

Dexamethasone: Dexamethasone inhibits placental estrogen synthesis by reducing secretion of the precursor, dehydroepiandrosterone sulfate, from the fetal adrenal glands[197,198]. An early observational study of 10 affected women from Finland suggested a beneficial effect with reduced serum estriol and estradiol levels and symptomatic improvement in all cases. In addition liver biochemistry, including the serum bile acid level, was improved and symptoms did not recur on cessation of treatment[199]. However, this was not supported by subsequent studies[189,200,201].

In addition to the conflicting reports of efficacy, there are concerns over safety. Dexamethasone has been widely used to promote fetal lung maturity and is reported to be safe in this context. However, it crosses the placenta easily, and animal and human data suggest that repeated high doses are associated with decreased birth weight[202] and abnormal neuronal development[203].

Rifampicin: Although there are no published studies reporting the use of rifampicin in ICP, it has been used with good results in several other liver diseases, including gallstones and primary biliary cirrhosis[204-206]. In these studies treatment with rifampicin resulted in significant decreases in serum levels of transaminases and total bile acids, as well as an improvement in pruritus, suggesting that it might also be useful in the treatment of ICP. A recent study investigating the molecular mechanism by which rifampicin works has shown that it enhances bile acid detoxification, an effect that is complementary to the up-regulation of bile acid export induced by UDCA, suggesting that the two drugs used in combination may be more effective than monotherapy[204]. The authors are aware of several ICP cases that have not responded to monotherapy with UDCA, but have responded to combined treatment with rifampicin and UDCA.

Vitamin K: ICP is associated with a risk of malabsorption of fat soluble vitamins due to reduced enterohepatic circulation of bile acids and subsequent reduction of uptake in the terminal ileum. Therefore many clinicians opt to treat women with oral vitamin K to guard against the theoretical risk of fetal antepartum and maternal intra- or postpartum hemorrhage. However, there have been no studies to support or refute this practice.

Others: S-Adenosyl-L-methionine (SAMe) is the principal methyl group donor involved in the synthesis of phosphatidylcholine, and therefore, it influences the composition and fluidity of hepatic membranes and hence biliary excretion of hormone metabolites[207]. It reverses estrogen-induced impairment of bile flow in rats[208-210]. Furthermore, in a human study of estrogen-induced cholestasis in women with a history of ICP, SAMe was shown to prevent ethinylestradiol-induced elevations in AST/ALT, bile acids and bilirubin[211]. Early studies of SAMe in the treatment of ICP reported that it improved both symptoms and biochemistry[212, 213], and these findings were confirmed by a subsequent placebo controlled study in which 15 women were treated with high dose SAMe (800 mg/d iv)[214]. However, a double-blind, placebo-controlled trial showed no improvement in symptoms or biochemistry following treatment with SAMe[215]. Finally, the efficacy of SAMe has been compared to that of UDCA, combination therapy with UDCA and SAMe, and placebo in one study[183]. Women treated with SAMe had a larger reduction in pruritus score and biochemical parameters than women in the placebo group, but this was not as large in the UDCA group. Furthermore, treatment with a combination of UDCA and SAMe was more effective than SAMe alone in reducing bile acid levels. Some patients have reported problems with peripheral veins following prolonged intravenous administration[215]. No other adverse maternal or fetal effects have been reported and SAMe seems to be well tolerated.

Cholestyramine is an anion exchange resin which acts by binding bile acids in the gut, thereby inhibiting the

enterohepatic circulation and increasing fecal excretion of bile acids. There have been several studies suggesting that cholestyramine is effective at reducing pruritus in ICP[216,217]. However, it has no effect on serum bile acid levels or other biochemical markers of cholestasis[216]. Furthermore, it may reduce the intestinal absorption of fat-soluble vitamins, thus depleting the levels of vitamin K and increasing the risk of hemorrhage for the mother and fetus[218]. Cholestyramine is therefore no longer considered a first-line therapy for ICP.

Guar gum is a dietary fiber that acts in a similar manner to cholestyramine. One small study has reported the use of guar gum in the treatment of ICP and reported no effect on serum bile acids or bilirubin and only a minimal reduction in pruritus score[219]. Subsequently, a randomized controlled trial has shown that guar gum is no more effective than placebo in improving pruritus or reducing serum bile acids[220].

Peroral activated charcoal has been shown to reduce serum bile acids in seven of nine women treated in one study. However, there was no improvement in symptoms[221].

Topical treatment with aqueous cream with 2% menthol is of value in the relief of pruritus, but does not improve biochemical abnormalities.

PROGNOSIS

Most women have no lasting hepatic damage, but ICP recurs in the majority of cases, with variations in intensity in subsequent pregnancies[10,222]. Recurrence is less likely following multiple pregnancy. Women with a history of ICP may also develop symptoms if taking the combined oral contraceptive pill or in the second half of the menstrual cycle[31]. Long-term follow-up studies have shown an increased risk of gallstones, non-alcoholic cirrhosis and pancreatitis, hepatitis C and autoimmune hepatitis[223,224].

CONCLUSION

ICP is a relatively common cause of hepatic impairment in pregnancy. It has a complex etiology with genetic, endocrine and environmental components. ICP causes maternal pruritus with impaired liver function and raised serum bile acids. The maternal cholestasis is transient with postnatal resolution, although affected women have increased rates of hepatobiliary disorders in later life. ICP is associated with adverse fetal outcomes. The risk of meconium-stained liquor, fetal asphyxia and spontaneous preterm delivery is greater in pregnancies with more marked elevations in maternal serum bile acid levels. The condition is also associated with IUD. The most effective pharmacological therapy for improvement of maternal symptoms and biochemical abnormalities is UDCA, and this has also been shown to reduce placental abnormalities and to improve placental bile acid transport in *in vitro* studies. Fetal outcomes are improved with a variety of strategies of active management, although the most effective intervention has not currently been established. A common practice is induction of labour at 37-38 wk of gestation with the aim of reducing the risk of IUD as many deaths occur at later gestations. Large therapeutic trials are required to establish which specific drug treatments or management strategies are effective at reducing the rates of adverse fetal outcomes.

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EDITORIAL

Safety of anti-tumor necrosis factor therapy in inflammatory bowel disease

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Abstract

Inflammatory bowel disease (IBD), in particular Crohn's disease refractory to conventional therapy, fistulizing Crohn's disease and chronic active ulcerative colitis, generally respond well to anti-tumor necrosis factor (TNF) therapy. However, serious side effects do occur, necessitating careful monitoring of therapy. Potential side effects of anti-TNF therapy include opportunistic infections, which show a higher incidence when concomitant immunosuppression is used. Furthermore, antibody formation against anti-TNF is associated with decreased efficacy and an increased frequency of infusion reactions. The hypothesis of a slightly increased risk of lymphomas in IBD patients treated with anti TNF-therapy is debatable, since most studies lack the specific design to properly address this issue. Alarmingly, the occurrence of hepatosplenic T-cell lymphomas coincides with combined immunosuppressive therapy. Despite the potential serious side effects, anti-TNF therapy is an effective and relatively safe treatment option for refractory IBD. Future research is needed to answer important questions, such as the long-term risk of malignancies, safety during pregnancy, when to discontinue and when to switch anti-TNF therapy, as well as to determine the balance between therapeutic and toxic effects.

Key words: Anti-tumor necrosis factor; Biologics; Inflammatory bowel diseases; Crohn's disease; Infliximab

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INTRODUCTION

Inflammatory bowel disease (IBD), including Crohn's disease and ulcerative colitis, is an idiopathic chronic relapsing inflammatory disorder of the intestinal tract[1]. The chronic and relapsing course of disease makes IBD a disabling disease that is complex to treat. Conventional therapy, including corticosteroids and thiopurines, is aimed at control of inflammation but does not appear to change the natural course of disease. Moreover, many patients become refractory to conventional therapies during the course of disease.

Infliximab was introduced in the late 1990s as the first result in the development of biologic therapies, and changed the therapeutic potential in IBD dramatically. Anti-tumor necrosis factor (TNF) therapy is currently used for the treatment of corticosteroid-refractory, active, corticosteroid-dependent, fistulizing Crohn's disease, as well as refractory ulcerative colitis[2,3]. Anti-TNF therapy is remarkably effective in patients who do not respond to conventional treatment. However, the use of biologics is associated with significant, but rarely, fatal complications, leading to serious concerns about safety and long-term consequences (Table 1). This review will discuss the current knowledge and safety issues as well as future directions for the role of anti-TNF therapy in the treatment of IBD.

SIDE EFFECTS OF BIOLOGIC THERAPY

Infections

The immunosuppressive effect of currently used biologics leads to an increased risk of specific infections during therapy. Most commonly, these infections arise from the upper respiratory tract and the urinary tract. Forty-eight patients had an infectious event and

Table 1 Side effects associated with anti-TNF therapy

Side effect	Example
Infections	Tuberculosis, histoplasmosis
Antibody formation	Antibodies to infliximab, antibodies to adalimumab
Infusion reactions	Anaphylaxis, delayed-type hypersensitivity
Autoimmunity	Antinuclear antibodies
Malignancies	Hepatosplenic T-cell lymphoma
Demyelization	Guillain-Barré syndrome
Abnormal liver function tests	Hepatitis, cholestatic disease
Cardiac abnormalities	Heart failure
Skin eruptions	Psoriasiform dermatitis

20 patients had a serious infection (an infection that requires antimicrobial therapy or hospitalization) during anti-TNF therapy, including fatal sepsis in two patients, in 500 Crohn's disease patients receiving infliximab[4]. In contrast, the CHARM study included 854 Crohn's disease patients, 517 of whom received adalimumab[5], and infectious adverse events occurred in 36%-44% of these patients. Serious infectious adverse events occurred in 2.7% of patients, and both types of adverse events were comparable to those in the placebo group. Serious infectious complications occurred in six of 216 patients (3%) treated with certolizumab[6].

Serious infections during anti-TNF therapy include the reactivation of latent tuberculosis. The increased awareness of this complication has led to a decrease in the number of reports of tuberculosis during biologic therapy. The risk of reactivation of latent tuberculosis was increased by seven-fold when the screening recommendations were not completely followed, as demonstrated by the Spanish national registry for anti-TNF therapy in rheumatoid arthritis[7]. After initiation of guidelines for tuberculosis screening prior to anti-TNF therapy, the rate of tuberculosis decreased by 78% in this registry[8]. Latent tuberculosis was identified by positive skin test and/or fibrotic lesions on chest X-ray in 16 patients in a single center cohort study including 734 IBD patients receiving infliximab. After chemoprophylaxis, none of these patients developed tuberculosis during infliximab therapy[9]. These findings suggest that the current treatment guidelines are indeed effective in preventing reactivation of latent tuberculosis. From 2001 to 2006, 130 patients with tuberculosis during anti-TNF therapy were reported in the USA[10]. The most important risk factor for disease reactivation was concomitant immunosuppressive therapy. Ominously, 34 patients in this group demonstrated a negative tuberculin skin test prior to anti-TNF therapy. Currently, every patient undergoing anti-TNF therapy should be screened by a careful medical history revealing any tuberculosis contact, followed by a chest X-ray and tuberculin skin test. As mentioned, this test is controversial due to reader variability and false-negative results. The recent T-cell-based interferon-γ assay seems more reliable with better sensitivity and specificity than the skin test, as shown in a group of 97 rheumatoid arthritis patients before initiation of anti-TNF therapy[11]. Patients with latent tuberculosis should start with chemoprophylaxis, for example isoniazid for 6 mo, during which anti-TNF medication can be introduced. Active tuberculosis should be fully treated before the start of anti-TNF therapy.

Data on the risk of fungal infections during anti-TNF therapy is limited. A database search identified 226 patients with fungal infections during infliximab therapy[12]. The most common pathogens were those causing histoplasmosis (30%), candidiasis (23%), and aspergillosis (23%). The majority of patients in this group were on concomitant immunosuppressive therapy (98%). Pneumonia was the most common manifestation of infection[12].

The reported risk of opportunistic infections in IBD patients treated with infliximab varies between 0.3% and 0.9%[13]. Interestingly, the risk of opportunistic infections dramatically increases when anti-TNF therapy is combined with additional immunosuppressive therapy, such as corticosteroids or thiopurine therapy[14]. The odds ratio for an opportunistic infection during infliximab therapy was 4.4, compared to 14.5 when combined with corticosteroids or thiopurines in 100 IBD patients with opportunistic infections, compared with a matched control group of IBD patients without opportunistic infections. The TREAT registry, which enrolled 6290 patients who received infliximab, showed that the increased risk for infections during anti-TNF therapy was associated with the use of corticosteroids and disease activity but not with the use of infliximab[15].

In summary, concomitant immunosuppression appears to be an important risk factor for infections during anti-TNF therapy. In daily practice, moderate to severe infectious complications prior to or during anti-TNF therapy require appropriate treatment of the infection before biologic therapy can be initiated or resumed safely.

Antibody formation

The monoclonal antibodies used for anti-TNF therapy frequently induce the formation of antibodies [antibodies to infliximab (ATI); antibodies to adalimumab (ATA)]. Sixty one percent of patients developed ATI in a study of 125 Crohn's disease patients who received on average four infusions of infliximab[16]. This development of ATI was associated with a shorter duration of response to therapy (35 d *vs* 71 d) and a higher rate of infusion reactions (relative risk 2.4)[16]. However, this correlation was not linear and did not predict infusion reactions in an individual patient. Importantly, immunosuppression in the latter study did decrease the formation of ATI.

Interestingly, recent data suggest that IBD patients who discontinued thiopurine therapy while continuing anti-TNF therapy did not show statistically significant clinical differences, compared to the group of patients receiving combination therapy[17]. This was demonstrated during a 2-year trial of 80 Crohn's disease patients. However, it should be noted that the infliximab

monotherapy group demonstrated lower infliximab trough levels and higher levels of C-reactive protein at 18-mo follow-up. We speculate that a prolonged follow-up period might have shown significant differences in the latter trends. ATI formation did not influence the pharmacokinetics of infliximab retreatment, although the authors discuss the influence of serum infliximab on the ATI assay in their paper, leading to an inability to draw firm conclusions[17]. Feagan *et al*[18] demonstrated that the efficacy of infliximab monotherapy was comparable to combination therapy with infliximab and methotrexate after 50 wk of treatment in Crohn's disease patients. Thus, although concomitant immunosuppression does reduce the formation of ATI, the clinical impact has recently been questioned. To further investigate the rationale for combination therapy with azathioprine and biologics, the SONIC trial included Crohn's disease patients who were naïve to immunosuppressive agents and biologic therapy with moderate to severe disease[19]. Patients were started on either azathioprine, infliximab, or a combination of both, and each group included 169 patients. At 26 wk, patients treated with infliximab monotherapy or infliximab plus azathioprine were more likely to achieve steroid-free remission and complete mucosal healing than those receiving azathioprine alone, whereas infliximab plus azathioprine was more effective than infliximab monotherapy. Further investigation in this field is warranted in order to guide patients in evidence-based choices to advise mono- or combination therapy.

Dosage and interval play a role in the development of ATI. For example, infliximab appears to be less immunogenic with increasing dose, as shown with different doses (1, 3 and 10 mg/kg) of infliximab in rheumatoid arthritis patients[20]. The immunological phenomenon of high-dose tolerance may explain this inverse dose-response correlation. Episodic treatment with anti-TNF therapy will also lead to an increased chance of developing antibodies to anti-TNF upon rechallenge. Therefore, scheduled maintenance rather than episodic therapy is recommended[21].

Adalimumab is a fully humanized IgG1 antibody to TNF and is considered less immunogenic than infliximab. The CLASSIC-2 trial demonstrated 2.6% antibody development in 269 patients receiving maintenance therapy for 56 wk[22]. All patients who developed antibodies in this study were not on concomitant immunosuppressive therapy. Yet, an ELISA was used for the detection of antibodies in this study. This technique has significant limitations due to the lack of discrimination between antibodies and anti-TNF medication[23]. This phenomenon may lead to underestimation of the true concentration of antibodies. Therefore, it is recommended that serum samples should be tested shortly before the next dose of anti-TNF in order to reduce the interference of anti-TNF medication[23]. A radioimmunoassay (RIA) is another technique to measure antibodies to anti-TNF medication. This technique measures specific high-avidity IgG antibodies against infliximab or adalimumab by an antigen-binding test[24]. The advantages of this assay are that it includes IgG4 antibodies, and it is more sensitive than ELISA due to a higher protein-binding capacity[23]. RIA measurements led to the detection of a higher percentage of patients who developed ATI or ATA when compared to previously reported findings[23]. Indeed, West *et al*[25] looked at 30 Crohn's disease patients who lost response to infliximab and were subsequently started on adalimumab. ATA were detected in five patients using RIA, four of these were non-responders to adalimumab. In this study, 17 patients were not on concomitant immunosuppression, and this subgroup included four patients with ATA. The authors concluded that ATA negatively influenced responses to adalimumab. In patients treated with certolizumab as maintenance therapy, 12% developed antibodies without concomitant immunosuppression, while 2% developed antibodies with immunosuppression[6].

Of interest, Aarden *et al*[23] demonstrated that low levels of anti-TNF, just prior to administration of the next dose, preceded the detection of ATI or ATA. Given the need for prevention of antibody formation during maintenance therapy and the technical challenges in the measurement of antibodies, assessment of trough levels rather than antibody development could be used as a biomarker for therapy adjustment. Therapeutic drug monitoring to guide therapy efficacy has not yet been elaborated.

Infusion reactions

The overall percentage of infusion reactions with infliximab was 6.1% in a group of 165 IBD patients[26]. These reactions included a burning sensation, itching, erythema, and pain. The estimated occurrence of serious adverse reactions (including shortness of breath, hypotension, or stridor) was 1.0%. In the latter study, all reactions were effectively treated[26]. Prophylactic antihistamines or a single-dose of hydrocortisone can be considered. In addition, patients who are off treatment for more than 4 mo are more susceptible to developing ATI and infusion reactions and should preferably receive these precautions. Most patients can be rechallenged after the appropriate precautions. Rarely, a genuine allergic reaction occurs, which is characterized by shortness of breath and urticaria[26]. These reactions are IgE-mediated and due to mast cell or basophil degranulation. In this case, the infusion should be stopped and switching to a different anti-TNF agent, such as adalimumab[27].

Delayed hypersensitivity-like reactions occur 3-14 d after anti-TNF therapy. Arthralgia and muscle ache are the most common symptoms[26]. It is believed that immune complex depositions take place and cause the latter symptoms[27]. Most patients with a large interval after the first administration of anti-TNF therapy develop delayed hypersensitivity upon rechallenge. Symptoms can be treated by acetaminophen and high-dose corticosteroids; symptoms usually resolve after 1-2 wk[27]. This group of patients will benefit from switching to a fully humanized anti-TNF therapy since poor responses to infliximab can be expected due to circulating ATI[16].

As a rule, adalimumab and certolizumab are administered subcutaneously. Injection site reactions, attributed to local irritation, were observed in 4% during adalimumab and 3% during induction therapy with certolizumab[5,28]. However, in our experience, injection site reactions are a frequently reported bothersome side effect of long-term adalimumab use. Injection site reactions regarded as a direct toxic effect do not improve following administration of antihistamines.

Autoimmunity

Anti-TNF therapy leads to cell lysis, in turn inducing circulating DNA and cell fragments, followed by the formation of autoantibodies such as antinuclear antibodies (ANAs). The percentages of autoantibodies differ depending on the therapy administered. Antibodies developed in 8% of certolizumab-treated patients after 6 mo, while infliximab led to > 50% of patients testing positive for autoantibodies[29,30]. Antibodies against double-stranded DNA were observed in 33% of 43 ANA-positive Crohn's disease patients receiving infliximab[30]. The development of antibodies is not limited to IBD patients or the use of infliximab; adalimumab induced ANAs in 45% of patients after 24 wk of treatment, and for infliximab, this number was 63% in a group of 91 rheumatoid arthritis patients[31]. Forty-one of 43 rheumatoid arthritis patients receiving infliximab and methotrexate demonstrated ANAs on at least one occasion, suggesting that concomitant immunosuppression does not reduce the formation of autoantibodies[32]. Furthermore, the formation of autoantibodies did not affect the efficacy of anti-TNF therapy and did not predispose to autoimmune diseases, in particular, systemic lupus erythematosus.

Malignancies

TNF plays a role in apoptosis and tumor suppression; it is believed that interference with these pathways can potentially lead to an increased risk of malignancies. However, the small size of clinical trials relative to the low incidence of lymphomas, the underlying risk of malignancies due to IBD, and the concomitant immunosuppressive therapy make it difficult to estimate the true effect, if any, of anti-TNF therapy on the genesis of malignancies in IBD patients. A large population-based study including 47 000 Crohn's disease and ulcerative colitis patients showed a standardized incidence ratio for lymphomas of 1.0 and 1.3 for ulcerative colitis and Crohn's disease, respectively[33,34]. The odds ratio for all types of cancer was 3.3 in a pooled analysis of both Crohn's disease and rheumatoid arthritis patients receiving infliximab or adalimumab[35]. Ten lymphomas were detected in 3493 patients receiving anti TNF therapy, whereas no lymphomas were reported in the control group. However, rheumatoid arthritis is associated with an increased risk of lymphomas, the latter being a disputed association in Crohn's disease[36,37]. The TREAT registry demonstrated that there was no significant increase in the relative risk for lymphoma (1.3) in 3272 patients treated with infliximab[15].

IBD patients undergoing immunosuppression are at increased risk for infections, including Epstein-Barr virus, which in turn may be associated with an increased risk of developing lymphomas. Seven of 18 lymphomas detected in IBD patients were Epstein-Barr-virus-positive, five patients in this group underwent therapy with azathioprine or 6-mercaptopurine[38]. However, the use of anti-TNF agents was not recorded in this study.

Hepatosplenic T-cell lymphoma is a rare type of non-Hodgkin's lymphoma with an aggressive and mostly fatal outcome. Until recently, 16 patients, mostly Crohn's disease patients who were exposed to infliximab, developed this type of lymphoma[39]. All patients received concomitant immunosuppressive therapy with thiopurines, and most patients also received corticosteroids. Of interest, three patients in this group received adalimumab, including two patients who previously received infliximab. It is alarming that nine cases were reported in the last 2 years, although increased awareness and subsequent reporting might play a role in this recent increase. Currently, it is unclear whether infliximab, thiopurine therapy, concomitant immunosuppressive therapy, the underlying disease, separately or in combination, are risk factors for the development of these lymphomas.

Taken together, the hypothesis of a slightly increased risk of lymphomas in IBD patients treated with anti TNF-therapy is debatable. Most studies lack the specific design to properly address this issue. The relative contribution of many risk factors for the development of lymphomas remains to be determined, such as the duration of anti-TNF therapy, concomitant immunosuppressive therapy, and the activity of the underlying disease.

Pregnancy and biologic therapy

Large-sized antibodies do not pass the placenta in the first trimester of pregnancy, but placental transfer is indeed possible in the second and third trimester of pregnancy. However, infliximab was not detected in breast milk[40,41]. To date, limited data are available to address the safety of anti-TNF medication during pregnancy. Live births occurred in 67%, miscarriages in 15%, and therapeutic terminations in 19% in a series of 96 pregnant patients receiving infliximab for either Crohn's disease or rheumatoid arthritis[42]. These results are comparable to Crohn's disease patients not receiving infliximab. However, it should be noted that most women stopped infliximab after conception. The TREAT registry reported 66 pregnancies including 36 during infliximab infusions[15]. The number of miscarriages and neonatal complications were similar in the infliximab-receiving *versus* infliximab-naïve patients. In another study of 10 pregnant Crohn's disease patients intentionally receiving infliximab during pregnancy, all had live births, of which three infants were premature and one had a low-birth weight[43]. Infliximab was detectable in newborns from 2 to 6 mo after delivery in a group of five mothers who were followed from the sixth month of pregnancy until

after delivery[44]. The decreasing levels of infliximab in newborns despite continuous breastfeeding do suggest placental transfer rather than transfer *via* breast milk. According to the FDA drug safety classification, infliximab is a drug without documented human toxicity, and is therefore considered category B.

Data on the use of adalimumab is limited, although case reports do not show adverse effects after the use of adalimumab during pregnancy[45,46]. No increased risk for adverse pregnancy outcomes was observed in a prospective cohort including 30 pregnant adalimumab-exposed rheumatoid arthritis patients, compared to a control group. A similar outcome was detected for an additional 66 pregnant patients exposed to adalimumab who did not meet the study cohort criteria[47].

Until now, the use of infliximab and possibly adalimumab does not appear to lead to an increased risk for fecundity, pregnancy, or fetal development. The available data on toxicity and long-term effects during pregnancy and in newborns are limited, therefore, a restrictive approach of using anti-TNF therapy prior to and during pregnancy seems appropriate. However, it is also important to realize that active IBD is documented to be detrimental to fecundity and pregnancy, and active disease can potentially do more harm to the embryo, fetus and mother than anti-TNF therapy.

Other safety issues

Neurological disorders following anti-TNF therapy have been described. Nineteen cases of demyelinating events following administration of anti-TNF agents were revealed in a review of the Adverse Events Reporting System of the Food and Drug Administration[48]. The latter observation was associated with a variety of neurological symptoms, including paresthesia, cognitive dysfunction, ocular symptoms, difficulty walking, incontinence, and hemiparesis[48]. Most, but not all, patients demonstrated partial or full recovery. Furthermore, nine patients on infliximab and one patient on adalimumab developed Guillain-Barré syndrome[49]. Also, optic neuritis was described in eight patients receiving infliximab and in two patients receiving adalimumab[50].

Abnormal liver function tests are associated with infliximab therapy. These abnormalities include cholestatic disease[51] as well as hepatitis-like syndromes[52]. Five patients receiving infliximab for Crohn's disease (one), rheumatoid arthritis (three) and psoriatic arthritis (one) developed liver disease, including one with autoimmune hepatitis and one with cholestatic liver disease leading to liver failure[53]. In addition, mildly elevated liver enzymes do occur, and it is recommended that anti-TNF infusions are stopped when these increases exceed three times the upper limit of normal in the case of alanine aminotransferase[54]. Abnormal liver functions tests generally return to normal after discontinuation of anti-TNF therapy. Reactivation of viral hepatitis[55,56] has been described in patients treated with anti-TNF therapy. Therefore, it is advocated that in high-risk groups, patients receiving anti-TNF therapy should be screened for hepatitis B prior to the initiation of therapy, and if positive, nucleoside analogs should be prescribed prior to the start of biologic therapy[56]. Interval monitoring of serum aminotransferases and viral load is recommended[56].

Data on the use of anti-TNF therapy in HIV-positive patients are limited. No clinical adverse effects or changes in CD4 count and viral load were detected in eight patients with rheumatic disease that were followed during their therapy with infliximab or etanercept. In this group, five patients received concomitant methotrexate, and five patients were using highly active antiretroviral therapy[57].

Dermatological symptoms have been reported as a side effect of infliximab therapy. 150 patients developed a wide variety of skin eruptions in a single-center cohort study including 734 infliximab-treated patients[58]. The majority of these patients (61%) were diagnosed with psoriatic dermatitis. Most skin lesions responded well to topical corticosteroids.

Anti-TNF therapy can lead to an increase in the rate of heart failure with an increased risk of death. Worsening of congestive heart failure was reported through the FDA's MedWatch in a number of postmarketing reports. Of 47 reported cases, 38 were new and nine were exacerbations[59]. Therefore, its use is contraindicated in patients with class Ⅲ-Ⅳ New York Heart Association congestive heart failure.

FUTURE DIRECTIONS

The potential risk for malignancies and infections during anti-TNF therapy appears strongly increased with concomitant immunosuppressive therapy, such as thiopurines. Therefore, risk stratification in order to reduce side effects in IBD patients requiring immunosuppressive therapy will become an important part of long-term treatment in these patients. For example, previous and latent infections (Epstein Barr virus, tuberculosis, and hepatitis B), previous malignancies and comorbidity should be taken into account to decide whether to withdraw immunosuppressive agents in order to reduce long-term side effects and maintain remission in IBD patients. Studies that address the reduction of immunosuppressive agents, like the withdrawal of thiopurines and continuation of infliximab in the study by Van Assche *et al*[17], provide valuable data for the potential reduction of concomitant therapies in IBD patients. Future trials to determine the effects of monotherapy *versus* combination therapy, such as anti-TNF, thiopurines, and methotrexate, will be important to guide this strategy. Furthermore, goals of therapy need to be defined. Should physicians aim for more aggressive therapy to ultimately achieve mucosal healing while increasing the risk of side effects, or should clinical remission remain the goal? Future research will help to provide patients with optimal therapy leading to quiescent disease and minimal side effects.

CONCLUSION

Anti-TNF therapy is a robust and effective therapy for

refractory IBD patients. The side effects can be severe, therefore, careful consideration and monitoring can partially prevent damage. Abscesses and opportunistic infections should be treated, and screening for tuberculosis as well as hepatitis B and HIV in high-risk patients is mandatory before the start of treatment. Antibody formation against anti-TNF agents can be prevented with concomitant immunosuppressive therapy, and the majority of infusion reactions due to infliximab can be prevented with antihistamines and corticosteroids. The risk of lymphomas requires careful consideration before the start of biologic therapy. Information on anti-TNF therapy in pregnancy is limited, although no adverse effects have been reported so far.

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TOPIC HIGHLIGHT

Giada Pietrosi, MD, *Series Editor*

Clinical applications of hepatocyte transplantation

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Abstract

The shortage of organ donors is a problem worldwide, with approximately 15% of adult patients with life-threatening liver diseases dying while on the waiting list. The use of cell transplantation for liver disease is an attempt to correct metabolic defects, or to support liver function as a bridge to liver transplantation and, as such, has raised a number of expectations. Most of the available studies briefly reported here focus on adult hepatocyte transplantation (HT), and the results are neither reproducible nor comparable, because the means of infusion, amount of injected cells and clinical variability differ among the studies. To better understand the specific role of HT in the management of end-stage liver disease, it is important that controlled studies, designed on the principles of evidence-based medicine, be done in order to guarantee the reproducibility of results.

Key words: Hepatocyte transplantation; Metabolic diseases; Chronic liver disease; Liver failure; Stem cells

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INTRODUCTION

The shortage of organ donors is a problem worldwide, with approximately 15% of adult patients with life-threatening liver diseases dying while on the waiting list. Hepatocyte transplantation (HT) may therefore become a viable alternative treatment to liver transplantation (LTx) for these patients. From 1992 to date, several studies on adult human HT have been conducted in patients with acute or chronic liver failure, in an attempt to correct metabolic defects or support liver function as a bridge to LTx[1]. Hepatocytes are isolated from the patient's liver (autologous) or from discarded transplant organs (homologous). Other potential sources are livers obtained from non-heart-beating donors, marginal grafts (steatotic, liver trauma), and segment Ⅳ (with or without caudate lobe) from split-liver techniques, in which one liver is used for two recipients[2].

ISOLATION AND INFUSION TECHNIQUES

The isolation of hepatocytes must meet the standards of good manufacturing practice. The liver is digested by collagenase and the hepatocytes obtained are generally transplanted fresh or thawed after cryopreservation. Both types of cells seem to be efficient, although there is perhaps an advantage to using fresh cells. Although the liver and the spleen are the most reliable sites used, the peritoneal cavity has also been used for transplantation in patients with fulminant hepatic failure[3]. While the infusion route used for cell transplantation is usually the portal vein, the splenic artery or a direct splenic puncture have also been used. The choice of the organ to infuse should be based on the underlying liver architecture, which, in the case of cirrhosis, may limit the hepatocyte engraftment because: (1) there is diffuse and abundant extracellular matrix, i.e. a potential endothelial barrier for nesting; (2) the portosystemic shunts could favor the translocation of hepatocytes to the pulmonary circulation; and (3) the presence of portal hypertension may expose patients to the risk of portal thrombosis,

with the consequence of further deterioration of the existing liver function. Injection through the portal vein should then be reserved for correcting inborn metabolic errors, while the splenic artery should be considered for patients with a fibrotic liver. The splenic puncture poses too many risks for patients with splenomegaly and portal hypertension. For hepatocyte transplantation into the liver, up to 10^9 cells per treatment can been infused *via* the portal vein, either through an indwelling catheter into a branch of the inferior mesenteric vein or through a catheter placed transhepatically under radiographic control. During the infusion, it is essential to monitor the portal venous pressure to avoid the risk of portal hypertension. The hypothetical aim is to perform repeated cell infusions in order to provide approximately 5%-10% of total liver mass, though it is still not clear what constitutes the maximum number of liver cells that can be infused each time, how many infusions can be performed in total and what the required hepatocyte mass is, depending on the specific metabolic deficit and stage of chronic liver disease.

Transplanted hepatocytes engraft, proliferate and function metabolically, as shown by several animal models. Moreover, in humans, their capacity to engraft in the liver has been demonstrated in a female patient with acute liver failure who received 2.8×10^7 male hepatocytes through the splenic artery. Nested PCR for the Y chromosome was performed in the explanted liver 10 d after the infusion, showing an engraftment ratio of 1:4000[4]. The immunosuppression scheme resembles that used in whole-organ transplant, and is generally based on tacrolimus ± steroids.

CLINICAL STUDIES

Adult HT for metabolic liver diseases

Inborn errors of metabolism affect around 1 in 900 live births, and LTx is an accepted and successful treatment for liver-based metabolic disorders, with more than 90% of children achieving long-term survival[5]. The success of auxiliary LTx in humans[6] supports the observation in animal experiments that a relatively small amount of liver tissue can provide sufficient function to correct the underlying metabolic defects. The number of transplanted cells is between 5% and 10% of the liver mass, with a varying amount of cells depending on the use of fresh *vs* cryopreserved cells. About 27 children have received liver cell transplantation, through portal or umbilical vein, for inborn errors of metabolism. Among children with urea cycle disorders, three of them with ornithine transcarbamylase deficiency (OCT) had NH3 control and evidence of OCT activity on liver biopsy. A 3.5-year-old girl with argininosuccinate lyase (ASL) deficiency and psychomotor retardation received a total of 4.7×10^9 hepatocytes (divided into 11 infusions), with important ammonium level reduction, a 3% ASL activity on liver biopsy at 8 mo (undetectable at baseline), and evidence of engrafted male cells (12.5%) at 1 year[2,7-10]. A 9-year-old Crigler-Najjar type 1 baby achieved a 50% reduction of bilirubin after receiving 5% of the hepatic mass divided into three intrahepatic infusions over 24 h, and returned toward pre-transplant levels 2 years later, despite evidence of functioning, engrafted allogenic hepatocytes[12]. Five patients with homozygous familial hypercholesterolemia were transplanted with autologous (left lateral liver segment resected) genetically modified hepatocytes, with an *ex vivo* transduced low-density lipoprotein (LDL) receptor gene. In three of them, a more than 20% reduction in LDL-cholesterol was observed up to 28 mo after liver-cell transplantation (the longest sustained reduction rate reported in pediatric cases), but with evidence of a < 5% transgene expression at 4 mo[15]. Three children with factor Ⅶ deficiency showed an 80% reduction in exogenous factor Ⅶ replacement up to 6 mo after transplantation[11,16]. Intraportal HT had no benefit for two children with progressive familial intrahepatic cholestasis, but the failure was attributed to significant liver fibrosis found at the time of LTx[11]. Twelve patients, who had received HT as a bridge to transplantation, subsequently underwent elective orthotopic LTx (Table 1).

Table 1 Adult HT in metabolic liver disorders

	Patients (n = 27)	Range of viable[1] cells number	Outcome (died/LT)	References
Urea cycle (OTC/ASL/ASS: 5/1/1)	7	$1.9\text{-}4 \times 10^9$	1/4	[2,7-10]
Crigler-Najjar type 1	6	$1.5\text{-}7.5 \times 10^9$	-/3	[11-14]
Hyper-cholesterolemia	5	$1.0\text{-}3.2 \times 10^9$	-/-	[15]
Factor Ⅶ deficiency	3	$1.1\text{-}2.2 \times 10^9$	-/2	[11,16]
Others	6[2]	$3.2\text{-}7.5 \times 10^9$	-/3	[11,17,18,20]

[1]In a few cases several cell infusions were performed; in one patient up to 18 infusions; [2]Glycogenosis type 1 (n = 2); refsum disease (n = 1); progressive familial intra-hepatic cholestasis (n = 2); α-1-antitrypsine deficiency (n = 1).

Adult HT for chronic liver disease and fulminant hepatic failure

Twenty patients have received HT for chronic liver disease. The first human hepatocytes were autotransplants performed in 1992 in 10 patients with chronic liver disease, using the left lateral segment as cellular source[19]. Transplanted hepatocytes were detected in the spleen with Tc 99m labeling at 1 and 6 mo. The next 10 patients were treated mostly with intrasplenic artery infusion (in two cases, the infusion was intraportal) and had some improvement in encephalopathy, hepatic protein synthesis and renal function. Four of them died. A liver transplant recipient with acute graft dysfunction, who had received an intraportal infusion, developed portal thrombosis and died the same day (Table 2)[4,20-22].

Patients with fulminant hepatic failure (FHF) have the highest mortality while on the waiting list, with an estimated 10% survival. HT can potentially support liver function until an organ becomes available or the liver regenerates. In a 1994 study[3], fetal hepatocytes

Table 2 Adult hepatocyte transplantation in chronic and fulminant hepatic failure

	Patients	Viable cells range	Outcome died/alive/LT	References
Chronic liver diseases	20			
Autotransplant	10	1.7×10^7-6.0×10^8	/	[19]
Allotransplant	10		4/6/3	
Alcohol	5	/	2/3/-	[20]
α-1-antitrypsine deficiency	1	2.2×10^7	-/1/1	[4]
HCV related	1	7.5×10^6	1/-/-	[4]
Other	3[1]	5×10^8-2×10^9	1/2/2	[21,22]
FHF	22		13/9/7	
Viral (HSV, HBV)	6	1.2×10^8-3×10^{10}	3/3/2	[4,20,23]
Drug-related	10	2.8×10^7-3.9×10^{10}	8/2/2	[4,13,20,22,23]
Idiopathic	4	1.8×10^8-4×10^9	1/3/3	[20,22]
Other	2[2]	1.7×10^8-4.9×10^8	1/1/-	[1,20]

[1]Cryptogenic cirrhosis (n = 1); idiopathic fibrosis (n = 1); liver transplant recipient (n = 1); [2]Mushroom poisoning (n = 1); trisegmentectomy (n = 1).

(60×10^6/kg body weight) were injected in 10 patients intraperitoneally through a dialysis catheter. Three of them recovered, showing neurological improvement, and decreased ammonia and bilirubin levels just 48 h after the infusion. No complications were related to the procedure. Among the 22 patients who received adult HT (Table 2), 11 had splenic artery infusion, nine had portal vein infusion and two received both splenic and intraportal infusion. Nine patients had a complete recovery (seven of whom received LTx). Two patients with herpes simplex virus and one with hepatitis B virus-related liver disease died[1,4,13,20,22,23].

FUTURE PERSPECTIVES

Most of the studies done in this field still focus on adult hepatocytes for transplantation, because this type of hepatocyte is considered a potential resource for bridging to LTx. However, this emphasis should perhaps be tempered by two important considerations: (1) adult hepatocytes are scarcely available, since they are obtained principally from discarded organs that cannot be transplanted; and (2) adult hepatocytes have limited proliferative capacity.

Alternative cell sources with vast capacities to consider for clinical application are stem cells and stem-cell-derived hepatocytes. Fetal tissues are in fact already deemed by the scientific community to be a promising source of liver stem cells to be used for clinical purposes. In Europe (Italy included), a multicenter study is underway on the use of fetal neuronal cells for the treatment of degenerative diseases. A study published in 2000 showed a functional improvement of cognitive-motor abilities in patients with Huntington's disease after human fetal neuron transplantation[24]. Fetal liver cells have several advantages compared to adult liver cells: greater availability, proliferative capacity and plasticity, less immunogenicity, good adaptation and integration capacity, and greater resistance to cryopreservation and ischemia. Moreover, there are no reports of oncogenic transformation, at least 2 years after intrasplenic fetal hepatocyte transplantation, in animal models[25].

The use of cell transplantation for liver disease raises a number of expectations, though it is important that controlled studies designed on the principles of evidence-based medicine be done in order to guarantee the reproducibility of results. At the same time, before establishing the safest and most effective number of cells to be infused, an accurate method for quantifying the engraftment rate, associated with specific tests of hepatocyte functionality, should be developed. A strict selection of candidates, and stratification by clinical scores (e.g. Meld score), could finally help clinicians to better understand the specific role of HT in the management of end-stage liver disease.

CONCLUSION

The results available in the literature are neither reproducible nor comparable. The means of infusion, the amount of injected cells and the clinical variability differ among the studies. In addition, a well-defined protocol of clinical and biochemical monitoring has yet to be established. However, the partial correction of the inborn errors, the sustained engraftment of at least 1/8 of the infused hepatocytes, and the prolonged survival in pediatric patients with metabolic liver diseases are encouraging enough to consider adult HT an effective bridging procedure to LTx for this category of patients. As it can be seen from the cases summarized in Table 2, 40.9% of patients with FHF and 60% of patients with chronic liver disease benefited from the hepatocyte infusion because they survived, with or without LTx. Nevertheless, it is possible that patients who recovered could have done so by spontaneous remission of the disease. Otherwise, in the remaining half of the patients, it could be hypothesized that the reason HT was not effective was attributable to the paucity of cells injected (rather than the loss of hepatic function in liver failure), or to the immunosuppressive regimens used (based on those for whole organ transplantation), which were not optimal for guaranteeing the function of the transplanted cells.

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OBSERVATION

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Tropheryma whipplei infection

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Abstract

Whipple's disease was initially described in 1907. Over the next century, the clinical and pathological features of this disorder have been better appreciated. Most often, weight loss, diarrhea, abdominal and joint pain occur. Occasionally, other sites of involvement have been documented, including isolated neurological disease, changes in the eyes and culture-negative endocarditis. In the past decade, the responsible organism *Tropheryma whipplei* has been cultivated, its genome sequenced and its antibiotic susceptibility defined. Although rare, it is a systemic infection that may mimic a wide spectrum of clinical disorders and may have a fatal outcome. If recognized, prolonged antibiotic therapy may be a very successful form of treatment.

Key words: *Tropheryma whipplei*; Small intestinal malabsorption; Abdominal lymphadenopathy; Periodic acid-Schiff staining; Whipple's disease

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INTRODUCTION

Whipple's disease was first described in 1907. It required almost 100 years before the responsible organism, *Tropheryma whipplei* (*T. whipplei*) was cultivated, its genome sequenced and its antibiotic susceptibility defined[1-5]. Detailed and authoritative reviews regarding the disease have also recently appeared[6,7]. Whipple's disease is known to mimic a wide spectrum of medical conditions, and yet, only 1500 cases or so have been described to date in the literature. Most expert clinicians, including specialist gastroenterologists, never see a single case over the course of their entire careers, however this disease is a principal bacterial cause of chronic malabsorption. As such, recognition of Whipple's disease should not be minimized since timely treatment might impact on the outcome of this potentially fatal disorder.

ORGANISM AND HOST FACTORS

Whipple's disease often affects middle-aged Caucasian men (but not exclusively), causing weight loss, arthralgia, diarrhea, steatorrhea and abdominal pain. Occasionally, other atypical presentations may occur due to involvement of the heart, lungs or central nervous system. The responsible organism is rod-shaped and can be seen in many different ultrastructural forms present in cells and extracellular spaces[8,9]. Usually, the organism is detected within macrophages of the lamina propria of the small intestine and its lymphatic drainage. The organisms, however, may also occur in epithelial cells as well as cells of the immune system. Because of genetic heterogeneity, some strains are non-pathogenic or may cause atypical clinical presentations such as an isolated infectious form of endocarditis[10]. Using a polymerase chain reaction (PCR) method, researchers found *T. whipplei* occurring in the environment and it has been documented in sewage water, fecal material and in sewage plant workers without Whipple's disease[11,12]. There may be a selective immune defect in host T-cells (or macrophages) that leads to Whipple's disease, or alternatively, these immune defects may be secondary and caused by *T. whipplei* itself[13].

CLINICAL AND LABORATORY FEATURES

Table 1 displays common clinical and laboratory features of *T. whipplei* infection. In some cases, there is a "prodromal phase" with fever and isolated joint manifestations, including arthralgia, preceding any gastrointestinal symptoms[14,15]. These joint symptoms

Table 1 Clinical and laboratory changes in *T. whipplei* infection

Clinical and laboratory changes	%
Clinical	
Weight loss	90
Diarrhea	80
Joint pain	70
Abdominal pain	55
Lymphadenopathy	50
Skin hyperpigmentation	40
Neurological changes	30
Laboratory	
Low serum carotene	95
Low serum albumin	90
Anemia	75
Elevated sedimentation rate	70

may be migratory in type and rheumatoid-factor-negative. Large joints may be involved more often than small joints alone and there may be treatment resistance to antirheumatic drugs. Duodenal biopsies may be negative, but synovial fluid and biopsies examined using PCR, immunohistochemistry or electron microscopy may reveal the diagnosis[16]. Diarrhea, weight loss and malabsorption associated with low serum carotene may occur[14,15]. Anemia with an elevated sedimentation rate may develop. Peripheral edema with hypoalbuminemia and ascites (associated with protein-losing enteropathy) may develop later in the clinical course. Endoscopic changes may be noted in some, but not all, patients and have recently been illustrated by Armelao *et al*[16]. Essentially, duodenal folds appear thickened and erythematous and yellow-white plaques may be seen. Duodenal biopsies are still the basis for diagnosis in the majority of cases and have been illustrated well elsewhere[17]. The histological features can be readily appreciated on standard hemotoxylin-eosin-stained sections of mucosal biopsies as massive infiltration of the lamina propria with foamy macrophages. These macrophages contain the organism. A periodic acid-Schiff (PAS) stain will confirm the suspected diagnosis. Rarely, the infiltrate may be limited to the submucosa. Lamina propria plasma cells and lymphocytes are not increased; indeed, with extensive macrophage infiltration, they may appear to be decreased. Small collections of fat may also be present in the lamina propria (thus, the term intestinal lipodystrophy coined by Whipple) and the overlying villus epithelium may appear vacuolated because of fat accumulation[17]. In part, this may reflect obstruction of lamina propria lacteals and regional lymphatics by lymph node involvement[17]. After treatment, the bacilli may disappear and the macrophage numbers become reduced, but both may persist for years[17].

Approximately a quarter of patients with Whipple's disease develop neurological changes, and some, despite treatment, are irreversible[18,19]. Neurological change may be the initial clinical feature, and rarely may occur in isolation[19-21]. Cognitive manifestations, such as dementia, are common. Altered ocular movements may occur, including a progressive form of supranuclear ophthalmoplegia. Headache, psychiatric changes, focal or generalized seizures and ataxia are frequent. Even without neurological symptoms, cerebrospinal fluid infection may be defined by PCR analysis[22]. Ocular involvement may include uveitis, retinitis and optic neuritis with papilloedema[23]. Historically, the disorder has been recognized as a form of culture-negative endocarditis. Diagnosis by valve explantation has been recorded[10,24].

Laboratory diagnosis of *T. whipplei* infection is still largely based on duodenal biopsy. Foamy macrophages in the lamina propria are seen that are PAS-positive, but diastase-resistant. Possibly, this positive staining reaction is related to the inner membrane of the polysaccharide bacterial cell wall. A Ziehl-Nielsen stain (most typically used for mycobacteria species) is negative. Other sites, e.g. lymph nodes, may also yield a classic PAS-positive staining reaction in the macrophages. PCR has a high sensitivity and specificity but is not recommended for screening because healthy carriers with a positive PCR have been noted. Recent studies using quantitative PCR on saliva and fecal materials make a case for a role of PCR in initial evaluation[25], followed by more invasive biopsy evaluation. Immunostaining with specific *T. whipplei* antibodies may reveal the organism in PAS-negative tissues[26]. Other biomarker methods are being explored[27].

TREATMENT

Before antibiotic treatment, a fatal course was often recorded. Later, tetracycline was often used, but recurrence was common and more recent treatment recommendations have been based on antibiotics that are capable of crossing the blood-brain barrier. Recent recommendations suggest that a 2-wk course of intravenous ceftriazone to achieve high cerebrospinal fluid levels, followed by twice daily cotrimoxazole for 1 year is very effective[7]. Most recover completely, although central nervous system symptoms may not resolve[7]. Others have suggested trimethoprim-sulfamethoxazole twice daily for 1-2 years[6]. Interestingly, treatment may be successful even if the diagnosis is established many decades after the onset of symptoms[28].

If ceftriaxone hypersensitivity is evident, then induction has been recommended with penicillin, cephalosporins, carbapenems, or chloramphenicol[7]. As an alternative to long-term cotrimoxazole, combination doxycycline and hydroxychloroquine have been recommended[7].

Recurrent neurological changes in Whipple's disease have a poor prognosis, and use of interferon gamma therapy has been described[29].

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REVIEW

Importance of nutrition in inflammatory bowel disease

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Abstract

Inflammatory bowel disease (IBD) results from the interaction between an individual's immune response and precipitant environmental factors, which generate an anomalous chronic inflammatory response in those who are genetically predisposed. Various feeding practices have been implicated in the origin of IBD based on epidemiological observations in developed countries, but we do not have solid evidence for the etiological role played by specific food types. IBD is associated with frequent nutritional deficiencies, the pattern and severity of which depends on the extent, duration and activity of the inflammation. Nutritional support allows these deficiencies in calories, macro- and micro-nutrients to be rectified. Enteral nutrition is also a primary therapy for IBD, especially for Crohn's disease, as it allows the inflammatory activity to be controlled, kept in remission, and prevents or delays the need for surgery. Nutritional support is especially important in childhood IBD as an alternative to pharmacological treatment. This report discusses the complex relationship between diet and IBD.

Key words: Nutritional support; Inflammatory bowel disease; Enteral diet; Crohn's disease; Ulcerative colitis

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INTRODUCTION

Inflammatory bowel disease (IBD) encompasses a heterogeneous group of chronic diseases of unknown etiology, unclear pathogenesis and a systemic nature that cause inflammation of the digestive tract, and includes Crohn's disease (CD) and ulcerative colitis (UC), which are traditionally found at opposite ends of the disease spectrum. While UC is exclusively restricted to the large bowel, CD can virtually affect any segment in the digestive tract, and may even be accompanied by extraintestinal manifestations. All diseases in the group involve alteration of the immunological tolerance system of the digestive tract mucosa[1], triggered by a certain factor which gives rise to an inappropriate, serious and prolonged inflammatory response in genetically predisposed individuals[2,3]. The ultimate causes of IBD have not yet been identified, but epidemiological studies show differences in the rate of IBD in terms of age and onset, race and geographical areas[4,5]. The existence of environmental factors is therefore suggested, which are capable of substantially altering the appearance of CD and UC. Among these, smoking and appendicectomy are the most notably implied, but the possible etiological role in the disease played by oral contraceptives, perinatal and childhood infections, or infections caused by atypical mycobacteria and diet has also been highlighted.

Various dietary and nutritional factors have been suggested as being significant etiological factors both for CD and UC[6], but at the same time, and more importantly, nutrition itself has proven to be a central component in the treatment of the disease, both as a primary therapy and for correcting the various nutritional deficiencies shown by these patients[7]. This report addresses these matters through a literature review, adding certain recommendations for the nutrition management of patients with IBD in the light of the evidence available.

DIET IN IBD

IBD results from the interaction of three essential co-factors: genetic susceptibility, environment and the immune response of the individual[8]. Environmental

factors may include both the local microenvironment (enteric microflora), and the nutritional environment. We do not have definitive data to demonstrate that diet is a cause of CD or UC, but over the past few decades, numerous studies have highlighted the potential etiological role played by certain feeding practices, based on the proportional increase of the incidence of IBD in developed countries and the appearance of new feeding habits in these regions[4]. New lifestyles include new feeding habits in which the consumption of cow's milk by children, the consumption of high quantities of refined sugar and fat and the low consumption of dietary fiber, fruit and vegetables take precedence.

Several studies have shown that breastfeeding reduces the possible development of UC[9-11] and CD[11-13]. Even in the case of infants who were breast fed for a short period of time, the risk of CD was significantly increased compared to the group that was breast fed for a longer time[12]. The consumption of cow's milk has also been implicated in the etiology of IBD[14], and these patients were shown to have higher levels of serum antibodies against cow's milk protein compared to healthy controls[15], with a correlation between the levels of specific antibodies and the index of activity in the case of adults with CD[16]. The relationship between breastfeeding and IBD has not been observed in other studies but various assumptions provide explanations as to the protective mechanisms of breastfeeding against IBD including: protection provided by breast milk against gastrointestinal infections[17-19]; its ability to stimulate the development of the gastrointestinal mucosa and its immunological capacity in children[20-23]; or postponing contact with cow's milk and other allergens and potentially infectious agents. Recently, the possible etiological role of *Mycobacterium avium paratuberculosis* as being an infectious agent which causes CD has been suggested[24,25]; this organism, originating from infected cows, could be transmitted through the milk and resist pasteurization[26]. However, several arguments against the putative role of *M. avium paratuberculosis* in causation of CD have been given, such as the lack of epidemiological support for transmissible infection, the absence of therapeutic benefit of traditional antimycobacterial antibiotics, and the low incidence of IBD in developing countries[27].

New feeding habits involve a high consumption of sugar and refined carbohydrates. Since the 1970s, various studies have indicated the high consumption levels of these products in patients with IBD[28,29], to the extent that they are now considered a risk factor for CD[30-32] and UC[31,33-35]. Conversely, the consumption of citrous fruit, fruit juices and vegetables could lower the risk[36] of the development of both diseases[37-39], and a particular study even showed an inverse relationship between the consumption of bran and the onset of CD[40]. To date, it has been impossible to determine whether the potentially protective effect is due to the action of the fiber or to other micronutrients contained in fruit and vegetables. The utility of low refined carbohydrate diets in the treatment of CD has been suggested by several authors[32,34], although extensive clinical trials have not confirmed the benefits of this measure[41].

In recent years, special attention has been paid to the lipid components of the diet as triggers of IBD. Since the earliest epidemiological relationships were demonstrated between the consumption of partially hydrogenated fats (margarine) and granulomatous ileitis[42] and UC[43], various studies have shown that new consumption patterns, such as fast food, could be linked to an increased risk in the development of CD and UC[36,44]. In addition, the consumption of large amounts of monounsaturated and polyunsaturated fats are both associated with a higher risk of UC[45,46]. The observation that the Eskimos in Greenland, consumers of large quantities of n-3 polyunsaturated fatty acids (PUFAs) deriving from fish oils, had a low prevalence of IBD[47,48] led to the study of the anti-inflammatory properties of n-3 PUFAs[49], in comparison with pro-inflammatory n-6 PUFAs. The latter have been clearly implicated in the origin of IBD, given that they affect the arachidonic acid metabolism by increasing the production of leukotriene B4, with pro-inflammatory action. These discoveries have opened up new channels of knowledge regarding the ability of lipids in the diet to regulate inflammatory processes in different diseases, as they are the fundamental component of cell membranes, including those of lymphocytes, which orchestrate immune system responses[50].

Short-chain fatty acids (SCFAs), of which butyrate is the most representative, are particularly worthy of note and are generated during the colonic fermentation of dietary fiber and other unabsorbable carbohydrates. A quantitative SCFA deficiency or their oxidation by colonocytes have been implicated in the physiopathology of UC[51,52], and SCFA *in vivo* oxidation is also lower in affected patients[53].

With regard to the protein and calorie intake in the diet, some studies have suggested that the intake of proteins[46,54] and calories[54] might be higher in patients with IBD compared to controls, although these data have not been uniformly observed and we do not know whether these factors are a cause or a consequence of the disease.

Despite the data presented at this moment in time, we still lack solid evidence regarding the accountability of certain dietary components in the etiology of IBD, although the aforementioned data oblige us to consider that the changes in the composition and characteristics of the diet which typifies modern life have been accompanied by substantial changes in the epidemiology of IBD in developed counties. However, we must remember that, beyond diet, our current lifestyle also has other characteristics whose possible etiological role in IBD has not been studied in depth.

NUTRITIONAL DEFICIENCY IN IBD

From the earliest descriptions of the disease, IBD,

especially CD, has been traditionally associated with serious nutritional deficiency. The pattern and severity of malnutrition in IBD depends on the duration, activity and extent of the disease, with significant differences having been described between CD and UC, given that the involvement of the small intestine is accompanied by a higher incidence of protein-calorie malnutrition and deficiencies in specific nutrients[55]. Furthermore, CD presents considerable chronic deficiencies, whereas in UC, the nutritional status tends to be more preserved, although during the flares of activity of the disease and in cases of hospitalization, the deficiencies tend to be significant[56]. In accordance with the methods and criteria considered for diagnosis, between 20% and 85% of IBD sufferers have nutritional deficiencies with prominent calorie-protein malnutrition in CD and protein malnutrition in UC[57]. A high proportion of CD patients (between 25% and 80%) and UC patients (between 25% and 50%) present hypoalbuminemia during hospitalization[55,58], which may clinically manifest as weight loss.

The origins of malnutrition in CD are multifactorial, but dietary restrictions (due to intolerance of diet or therapeutic fasting) are the most important. Also included are: the increase in energy requirements[59-61], the malabsorption of nutrients in the case of extensive intestinal involvement, gastrointestinal losses and the interaction between nutrients and drugs. Furthermore, the underlying inflammatory mediators of the physiopathology of IBD[62], such as tumor necrosis factor (TNF)-α, and interleukins-1 and -6 can increase catabolism and lead to anorexia. Table 1 provides a summary of the causes of malnutrition in IBD[63].

Table 1 Causes of malnutrition in IBD (modified from García-Manzanares *et al*[63])

Decrease in oral intake	Restrictive diets, therapeutic fasting
	By the disease itself: diarrhea, abdominal pain, nausea and vomiting, *etc*
	Alteration in taste: due to drugs, vitamin and mineral deficiencies, pro-inflammatory mediators
	Anorexigenous effect of pro-inflammatory cytokines
Gastrointestinal losses	Diarrhea
	Rectorrhagia/hematochezia
	Loss of mucus and electrolytes
	Protein-losing enteropathy
Metabolic disorders	Increase in resting energy expenditure
	Enhanced fat oxidation
Increase in nutritional requirements	Inflammatory states
	Increased basal oxidative metabolism
	Infectious complications
	Post-surgery
Drug interaction	Corticoids and calcium reabsorption
	Corticoids and protein catabolism
	Salazopirine and folates
	Methotrexate and folates
	Cholestyramine and liposoluble vitamins
	Antimicrobials and vitamin K
	Anti-secretors and iron
Poor absorption of nutrients	Reduction of the absorptive surface: intestinal resection, enteric fistulas, hypertrophy of the villi
	Blind loops, bacterial overgrowth
	Poor absorption of bile salts in ileitis or resection

Although micronutrient deficiency in IBD is common, in most cases it does not tend to have any evident clinical manifestation, except with regard to iron, folic acid, and vitamin B12[57]. However, those micronutrients which have an impact on bone mineral density, thrombophilia or carcinogenesis are of significant clinical interest. Little is known about other micronutrient deficiencies in IBD in terms of their consequences, frequency and subclinical development, due to the lack of studies in this area. However, many of them could be involved in regulating immune response at different levels[64].

IBD patients show an increased loss of bone mass[65,66], which could lead to osteopenia and osteoporosis, and which in certain studies, affects up to half the number of patients with CD and UC[67,68] and contributes to an increased risk of fractures up from 40% to 60%[60]. Although multifactorial in origin, the action of certain pro-inflammatory cytokines (especially TNF-α) has recently been highlighted with respect to bone loss[67]. Aside from the chronic or recurring use of corticosteroids[69], age, the female gender, type of IBD, smoking and other hormonal and genetic factors also contribute to osteoporosis in IBD[67].

Folic acid deficiency observed in half the number of patients with IBD might be due to difficulties in swallowing (low-fiber diets), poor absorption or competitive inhibition by certain treatments, such as sulphasalazine or methotrexate[63]. The absence of folic acid has been related to the increased risk of colitis-associated carcinogenesis[55,70], as it has a protective effect against high-grade dysplasia and cancer in patients with long-term UC[71,72]. Folate deficiency is also linked to the increased incidence of arterial and venous thromboembolic events observed in CD and UC[73], due to hyperhomocysteinemia, a well-known inducer of hypercoagulability states. Both folic acid and vitamin B12 are essential co-factors in the metabolic route of homocysteine-methionine[74]. Between 20% and 60% of patients with CD and terminal ileitis are deficient in vitamin B12.

Other relevant nutritional deficiencies in CD are iron, zinc or selenium. Zinc is a vital component for the healing of wounds and its deficiency should be considered in the case of recurrent fistulous disease[75,76]. In addition, zinc is a co-factor of superoxide dismutase, which protects against cell damage caused by free radicals. Selenium is a co-factor of glutathione periodoxase[63]. Oxidative stress is one of the factors which perpetuates the inflammatory response in IBD[77], which is why a sufficient intake of antioxidant agents such as vitamins A, C, E and selenium is of extreme importance and has been inversely correlated with the plasma levels of pro-inflammatory agents[77,78].

Malnutrition has particularly serious direct consequences for patients with IBD. The scope thereof

depends on various factors, noteworthy being the age at which the disease begins and its activity. Delayed growth in children is the most frequent extraintestinal manifestation[79]; it is detected early and affects 75% of patients with CD and 10% of cases of UC[63]. Various pro-inflammatory cytokines, which are frequently high in IBD[80], are involved in the growth retardation and puberty of these children, as well as absorption deficiencies or increased catabolism. The objectives in the treatment of these patients should be aimed at acquiring knowledge of the inflammatory mechanisms and the control of their effects using immunomodulatory and biological treatments and at optimizing nutritional treatment[81], which frequently requires coordination among gastroenterologists, endocrinologists and nutritionists.

Calorie-protein malnutrition causes humoral and cellular immunodeficiency. Its effects on the intestine lower the efficiency of the mucosal barrier, lead to alteration of the functionality of the mucosa-associated lymphoid tissue and to a greater risk of infection by bacterial translocation. Hypoplasia of the intestinal villi perpetuates malabsorption and increases the risk of infections.

Metabolic bone disease develops silently in these patients, the origin of which is probably multifactorial[82]: steroids, lack of physical activity, deficiencies of calcium, vitamins and other micronutrients and alterations of the intestinal villi.

NUTRITION AS AN EFFICIENT PRIMARY TREATMENT IN IBD

Nutrition therapy should play a fundamental role in the clinical management of all patients with IBD. Its objectives are to correct macro and micronutrient deficiencies in frequently malnourished patients subject to increased oxidative catabolism, to reverse the physiopathological consequences of such deficiencies, and also to exert its own anti-inflammatory therapeutic effect.

Enteral feeding using formulas or liquids should always take preference over parenteral feeding, unless it has been completely contraindicated. If oral feeding were not possible, feeding the patient through a nasogastric or nasoenteric tube should be considered. The value and benefits deriving from its use are directly dependent on the geographical location of the disease, its extent and gravity and enteral feeding is therefore especially indicated for CD patients when the small intestine is affected, while there is no evidence which supports the use of enteral nutrition in the treatment of UC. We have very little data regarding the efficiency of enteral nutrition in CD that is exclusively confined to the colon, although its remission rates might not show any differences compared to other locations of CD[83].

Apart from the intake of calories, proteins and micronutrients, enteral nutrition using liquid formulas performs other primary therapeutic functions in CD[84]. In 1973, the therapeutic effect of enteral nutrition exclusively using basic formulas (amino acids with no antigenic capacity) was described for the first time in adults with CD resistant to other therapies[85], as similar remission rates were achieved to corticosteroids[86,87]. This ability to abate CD activity in both adults[83] and children[88], extends to efficiency in maintaining remission[89-91], allowing delay in the need for surgery or reintervention[92]. Furthermore, it is a safe treatment for which no significant adverse effects have been reported.

With regard to enteral nutrition formulas, no differences were identified between the efficiency of elemental diets and non-elemental formulas[87,93], which leads to the rejection of the previously held idea that a diet lacking in antigenic capacity could restore the altered intestinal immune response. In this respect, the therapeutic effect of enteral nutrition in CD seems to be independent from the nitrogen source used[55]. On the other hand, the fat composition of the enteral diet seems to be more important in terms of its therapeutic effect on CD[94], as this fat composition could be the key factor of the diet's therapeutic action on the disease[95]. This has been suggested by various studies, but results are difficult to interpret, which means that we do not know what the ideal fat content in enteral nutrition should be for the treatment of CD. Various studies have assessed the efficiency of supplements using n-3 PUFAs in maintaining patients with CD[96,97] and UC[98] in remission, showing that they might only prove effective for maintaining CD cases in remission, although more extensive studies are required in order to unequivocally establish the utility of these therapies. In any case, these treatments are safe and no side effects have been reported.

The precise mechanism of action through which enteral nutrition operates in CD is not well known, but it has been suggested that it could act by modulating the immune system's mucosa, regulating imbalances in the bacterial flora capable of precipitating inflammation[99,100], or by modifying the luminal content, thereby altering the expression of certain genes in the epithelium with an effect on the immune system of the mucosa, as well as reducing the exposure of the intestine to antigens.

In recent years, we have increased our knowledge of the immunoregulatory function of intestinal microflora and its possible participation in the physiopathology of IBD[101,102]. Alteration of the composition and function of intestinal microbiota could lead to increased stimulation of the intestinal immune system, epithelial dysfunction and greater permeability of the mucosa, and accordingly, the correct characterization of the components of these microflora and the definition of their functions are vital in order to consider probiotic treatment for IBD[103,104]. Probiotics have shown to be as effective as mesalazine in preventing relapses in patients with UC and in the treatment of pouchitis. Efforts have also been made to identify dietary components (prebiotics) which are capable of regulating the bacterial

composition, or which have a trophic effect on the intestinal epithelium. SCFAs (butyrate, propionate and lactate) result from the fermentation of fiber by bacterial species in the colon (*Bifidobacterium*, *Eubacterium* and *Lactobacillus*), and are an important metabolic substrate for colonocytes that promote the good functioning of the mucosa[105]. The anti-inflammatory effect of butyrate has been the most studied at different levels in the physiopathology of the inflammation[51,106], and it has been successfully tested as a treatment for patients with UC[107,108].

Parenteral nutrition is of scant therapeutic interest in IBD since diverse studies have shown that intestinal rest is not beneficial to control the disease[109,110]. Consequently, parenteral nutrition is not useful for the induction or maintenance of remission in CD, nor do we have any evidence to support its use in UC. It is also very expensive and poses an additional risk due to the use of venous catheters[111]. Its utility is therefore restricted to certain cases involving efforts to close enterocutaneous or other complicated fistulas in patients with fistulizing CD[112,113], the treatment of short bowel syndrome following extensive resections for CD, or when enteral feeding is impractical for other reasons.

PRACTICAL CONSIDERATIONS

IBD is an important risk for malnutrition. Nutritional support using liquid formulas should be considered as a primary treatment for all patients with CD and in serious cases of UC, but especially for children and for those who may require prolonged cycles of corticosteroids, such as the youngest patients, those who are corticodependent, or those who present other risk factors for osteoporosis. Enteral nutrition may be considered both as a primary treatment and as a supplement to other medication in order to achieve or maintain CD remission[91].

A rich and varied diet should be recommended for all patients with IBD during remission, which includes fruit and vegetables, meat, olive oil and fish, especially blue fish. There are no reasons to restrict insoluble fiber in the diet except in the case of significant intestinal stenosis or when irritable bowel syndrome might co-exist that does not respond to other therapies. We do not have any studies that support the restriction of fiber in the diet during flares of the disease but the consumption thereof could be temporarily restricted at this time.

Because of their calcium content, dairy products are especially recommended for these patients and milk should only be restricted in the case of lactose intolerance, substituted by other fermented products (yoghurts and cheese) or calcium-enriched soya-based products. Calcium and vitamin D3 supplements are also required during treatments with systemic steroids and with those with a greater local effect, such as budesonide or beclomethasone. Iron and folic acid deficiencies should be routinely monitored in patients with IBD due to their high occurrence. Deficiency in one or both micronutrients is the main cause of anemia in these patients and can be easily remedied. We should warn that ferritin is an acute phase reactant that increases during inflammation, which restricts its value as a marker of ferropenia in IBD. For treating iron deficiency in IBD, iron can be orally or intravenously administered; the latter is recommended in cases of active inflammation in CD, since oral supplementation might be of limited efficacy. The absorption deficiency of vitamin B12 contributes to anemia and hypercoagulability. The resection or involvement of the terminal ileum in CD requires vitamin B12 supplementation *via* the parental route.

Ileum actively participates in enterohepatic circulation, which refers to circulation of bile acids from the liver where they are produced, to the small intestine, where they aid in digestion of fats and other substances, back to the liver. In this way, the distal ileum is necessary for fat and fat-soluble vitamin absorption. CD patients frequently undergo resection of the terminal ileum, and if a large segment of bowel is removed, malabsorption of these lipid diet components may appear.

The prevention of therapeutic non-compliance in IBD also includes nutritional supplements to diet and medication. In complying with nutritional treatment, aspects such as flavor, presentation, tolerability to the food, its potential adverse effects (diarrhea, nausea), the patients' motivation and that of the healthcare professionals who attend them, are influential. Attention should be paid to the distribution of the doses during the day and to the simultaneous administration of other solid food, together with the preferences of the patients themselves[55].

A number of commercial supplements are available that provide nutritional support in IBD, are wide in variety in terms of composition and nutritional content, and have a diversity of flavors allowing personal choice. We must warn that, for palatability reasons, the elemental or semi-elemental formulas are more suitable for administration *via* a nasogastric tube. Feeding using a nasogastric tube may also be considered for patients with specific protein or energy intake requirements, which for different reasons, can not be satisfied by oral means, but they may be fed a nutritional supplement *via* a tube during the night.

As detailed above, guaranteeing a sufficient calorie and protein intake can be a complicated task that may require the involvement of nutritionists and dieticians. Mutual trust between the patients, their families and the health professionals is vital to ensure the sufficient level of motivation for the adequate long-term nutritional compliance required by a chronic disease. Enteral nutrition is considered the number one treatment for CD in children, as an alternative to immunomodulatory drugs, due to its excellent safety record and advantages concerning growth. In these cases, cooperation between the patient's family and the professionals who care for him or her are particularly important to guarantee correct nutritional support.

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High *miR-196a* levels promote the oncogenic phenotype of colorectal cancer cells

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Abstract

AIM: To analyze the relevance of the microRNA *miR-196a* for colorectal oncogenesis.

METHODS: The impact of *miR-196a* on the restriction targets *HoxA7*, *HoxB8*, *HoxC8* and *HoxD8* was analyzed by reverse transcription polymerase chain reaction (RT-PCR) after transient transfection of SW480 cancer cells. The *miR-196a* transcription profile in colorectal cancer samples, mucosa samples and diverse cancer cell lines was quantified by RT-PCR. Transiently *miR-196a*-transfected colorectal cancer cells were used for diverse functional assays *in vitro* and for a xenograft lung metastasis model *in vivo*.

RESULTS: *HoxA7*, *HoxB8*, *HoxC8* and *HoxD8* were restricted by *miR-196a* in a dose-dependent and gene-specific manner. High levels of *miR-196a* activated the AKT signaling pathway as indicated by increased phosphorylation of AKT. In addition, high levels of *miR-196a* promoted cancer cell detachment, migration, invasion and chemosensitivity towards platin derivatives but did not impact on proliferation or apoptosis. Furthermore, *miR-196a* increased the development of lung metastases in mice after tail vein injection.

CONCLUSION: *miR-196a* exerts a pro-oncogenic influence in colorectal cancer.

Key words: Micro-RNA; Cancer; Colorectal; *miR-196a*; Migration; Homeobox

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INTRODUCTION

Survival in colorectal cancer (CRC), one of the three most prevalent malignancies in western countries, is delineated by local recurrence, lymphatic and distant dissemination[1-3]. Molecular determinants occurring during the adenoma-carcinoma sequence of sporadic CRC include mutations in certain tumor-suppressor genes (*APC*, *DCC*, *Smad-2*, *Smad-4*, *p53*) and oncogenes (*K-ras*) that have been summarized by Fearon and Vogelstein[4-6]. However, as only 8% of CRCs harbor concomitant mutations of *APC*, *K-ras* and *p53*, it seems very likely that additional pathogenic alterations are instrumental in promoting progression and metastasis of colorectal cancer[7].

A recently discovered class of non-protein-coding small RNAs, microRNAs (miRNAs), extend our understanding of oncogenesis. miRNAs are endogenous small RNA molecules of 20-25 nucleotides length, regulating gene expression by inhibiting transcription,

inducing direct cleavage of the targeted mRNAs or blocking translation through their complementarity *versus* targeted mRNAs at 3' untranslated regions[8-13].

More than 50% of all known miRNA genes are located in cancer-associated regions or in fragile sites of the genome, indicating that miRNAs might play an important role in oncogenesis[14]. Supporting evidence is the close location of miRNAs, as *miR-196a*, in homeobox (*Hox*) gene clusters[14]. Hox proteins are major transcription factors that play a crucial role during embryogenesis, organogenesis and oncogenesis[15].

While some miRNAs can function as oncogenes, others act as tumor suppressors. Specific miRNAs, such as *let-7*, are under-expressed in cancer and function as tumor suppressors by regulating oncogenes in normal tissue. New evidence indicates that down-regulation of *let-7* transcription is a relevant step during oncogenesis which is significantly associated with shortened postoperative survival in lung cancer[16-18]. *Let-7* negatively regulates the expression of oncogenes *Ras* and *Myc* by targeting their mRNAs for translational repression in diverse malignancies[19].

In contrast, over-expressed miRNAs, such as *miR-17-92*, function as oncogenes promoting cancer development through inhibition of tumor suppressor genes. The expression of miRNA *miR-17-92* is significantly increased in small-cell lung cancer[20]. Interestingly, the known targets of *miR-17-92* include the two well-known tumor suppressor genes, *PTEN* and *RB2*[21].

The miRNA *miR-196a*, encoded at three locations in the mammalian Hox clusters A, B, and C, depicts evolutionarily conserved complementarity to mRNA of *HoxB8*, *HoxC8*, and *HoxD8*[22]. Interestingly, *miR-196a*-directed cleavage of *HoxB8* was detected in mouse embryos, and additional *in vivo* experiments revealed a down-regulation of *HoxB8*, *HoxC8*, *HoxD8* and *HoxA7* in mammalian cells. These results indicate a miRNA-mediated regulation of *Hox* gene expression during vertebrate embryogenesis[22].

Matching these observations, Hornstein and colleagues describe that *miR-196a* acts upstream of *HoxB8* and *sonic hedgehog* (*Shh*) *in vivo* during limb development[23]. Analyzing the miRNA expression pattern in pancreatic adenocarcinoma by large-scale miRNA chip analyses, Croce and colleagues found that 75% of tumors expressed *miR-196a* at a high level, predicting poor patient survival and linking *miR-196a* to human oncogenesis (14.3 mo *vs* 26.5 mo)[24].

As we had previously investigated the relevance of *Hox* genes for gastrointestinal cancer progression and observed a tumor-suppressive function of high *HoxC8* expression levels, we hypothesized that *miR-196a* might exert a pro-oncogenic influence in human cancer cells.

MATERIALS AND METHODS

Cell culture and human tissue

The human colorectal cancer cell lines SW480, SW620 and HT29 and the human gastric cancer cell line Snu16 were cultured in RPMI-1640 (Invitrogen, Germany) supplemented with 10% FCS, 100 U/mL penicillin, 100 μg/mL streptomycin (Cambrex, Germany) and 1 mmol/L L-glutamine (Invitrogen, Germany).

Colorectal cancer and mucosal tissue has been collected from the resectate of seven patients undergoing elective surgery for colorectal cancer after obtaining patients' written informed consent and approval by the local ethics committee.

miRNA isolation and quantitative reverse transcription polymerase chain reaction (RT-PCR)

miRNA isolation was performed from four cancer cell lines, and from seven colorectal cancer and matching mucosal samples using the MirVana miRNA Isolation Kit according to the manufacturer's recommendations (Ambion, Austin, USA). HSA-*miR-196a* and *U6* primer sets were commercially acquired and applied for quantitative RT-PCR using the MirVana QRT-PCR miRNA Detection Kit with Super *Taq* Polymerase (Ambion). For amplification, an Applied Biosystems 7900 HT Fast Realtime PCR System (Applied Biosystems, Foster City, USA) was used.

miR-196a transfection

3×10^5 SW480 colon cancer cells were plated in a six-well plate and cultured as described before. SW480 cells were used, as they had the lowest *miR-196a* transcription levels (see below). *miR-196a* was commercially synthesized (MWG Biotech, Germany) and applied at different concentrations (0, 20, 40, 80, 160 and 240 nmol/L). Transfection was performed with Lipofectamine siRNAmax (Invitrogen, Carlsbad, CA, USA) according to the recommendations of the manufacturer. Cells were harvested 24-48 h after transfection and either applied in the functional assays, in a xenograft bioassay or collected for RNA/protein extraction, respectively.

Proliferation assays

6×10^3 transiently transfected SW480 cells (mock or 160 nmol/L *miR-196a*) were plated in 96-well plates and cultured as described above. The start of analyses was 24 h after transient transfection. The number of cells per well was determined daily by absorbance (MTT). Absorbance was quantified with an ELISA reader. Each condition was performed in quadruplicate.

Adhesion assay

For adhesion assays, SW480 cells were used. Transient transfection (mock or 160 nmol/L *miR-196a*) was performed 48 h prior to assay start. Ninety-six-well plates had been prepared with laminin (10 μg/mL, 30 min, room temperature, Sigma, Germany), fibronectin (10 μg/mL, 30 min, room temperature, Sigma) or PBS and were blocked with albumin (2%, overnight, 4℃, Serva, Germany), respectively. After trypsinization, 4×10^4 cells were seeded per 96-well and allowed to attach for 45 min. Thereafter, the medium and non-attached cells were removed. Each

well was washed twice with 100 μL pure RPMI-1640 cell culture medium. The number of attached cells per well was determined by luminescence assay (Celltiter-Glo Cell Viability assay; Promega, USA). Emitted luminescence was quantified with a luminometer. Each condition was performed in quadruplicate. For dose-dependent quantification of adhesion (0, 40, 80 or 160 nmol/L *mi*R-*196a*) non-modified 96-well plates were used.

Migration and invasion assays

For migration and invasion assays SW480 cells were used 48 h after transient transfection (mock or 160 nmol/L *mi*R-*196a*). Migration and invasion were assayed with 24-well HTS FluoroBlock Inserts in triplet approaches (8 μmol/L pore size; Becton Dickinson, USA). For invasion assays, membranes were covered with fibronectin in advance (10 μg/mL, 30 min, room temperature, Sigma) and blocked with albumin (2%, overnight, 4℃, Serva).

In brief, 4×10^4 cells were re-suspended in serum-free RPMI-1640 medium and added to the upper chamber. Consecutively, RPMI-1640 medium with 20% FCS and 100 ng/mL CXCL12 was added to the lower chamber. Chambers were incubated for 24 h at 37℃ in a humid atmosphere of 5% CO_2. After incubation, the amount of cell invasion and migration into the lower chamber was determined by luminescence assay (Celltiter-Glo, Cell Viability assay; Promega) according to the recommendations of the manufacturers. Emitted luminescence was quantified with a luminometer. Each condition was performed in triplicate.

Chemosensitivity

3×10^5 SW480 cells (mock or 160 nmol/L *mi*R-*196a*) were seeded per six-well plate. Twenty-four hours after plating, 5-fluorouracil (5-FU) (10 μg/mL), irinotecan (40 μg/mL), oxaliplatin (10 μg/mL), cisplatin (20 μg/mL) or placebo (1 × PBS) were added to the medium. The number of apoptotic cells was determined after 48 h by apoptosis assay. In brief, suspension cells were collected and adherent cells were trypsinized prior to fixation with 100% ethanol, stained with propidium iodide and analyzed by FACS without gating. Each condition was performed in quadruplicate.

Western blotting analysis

SW480 cells were harvested 2 d after transient transfection (mock or 160 nmol/L *mi*R-*196a*), washed twice with PBS (1 ×) and lysed in 2 × RIPA solution. For Western blotting analysis, 100 μg of protein was loaded on a 13% SDS-PAGE gel. After separation, the gel was transferred to a PVDF membrane (Roth, Karlsruhe, Germany). AKT protein was detected with a rabbit-anti-human antibody (1:1000, overnight, 4℃, rabbit-anti-human monoclonal antibody, pan AKT, 4685; Cell Signaling, Danvers, MA, USA). Phosphorylated AKT (pAKT) protein was detected with a rabbit-anti-human antibody (1:1000, overnight, 4℃, rabbit-anti-human monoclonal antibody, Phospho-AKT, 9267, Cell Signaling). MEK1/2 was detected with a monoclonal rabbit-anti-human antibody (1:1000, overnight, 4℃, rabbit-anti-human monoclonal antibody, 9122; Cell Signaling). pMEK1/2 was detected with a monoclonal rabbit-anti-human antibody (1:1000, overnight, 4℃; rabbit-anti-human monoclonal antibody, 9121; Cell Signaling). Alpha-tubulin was analyzed with a monoclonal mouse-anti-human antibody (T5168, 1:2000, overnight, 4℃, Sigma). The secondary antibodies used were goat-anti-rabbit (1:10 000, 1 h, RT, SC-2033, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and goat-anti-mouse (1:10 000, 1 h, RT, SC-2031, Santa Cruz Biotechnology). For visualisation the Roti Lumin systems 1 and 2 were applied (P79 and P80; Roth).

Lung metastases xenograft biosystem

Transient transfection (mock or 160 nmol/L *mi*R-*196a*) of SW480 was performed 48 h prior to assay start. 4×10^4 tumor cells were re-suspended in 0.2 mL pure RPMI-1640 medium and applied for induction of lung metastases in 7-8-wk-old nod-Scid mice. Nod-Scid mice were radiated with 1.8 Gy 1 d prior to intravenous injection (tail vein) of tumor cells. Lung tumors grew for 7 wk before the animals were sacrificed. Thereafter, lungs were resected and tumor nodules quantified manually using surgical magnifying glasses.

RNA isolation and semiquantitative RT-PCR

RNA isolation was performed using the Qiagen RNeasy Kit according to the manufacturers recommendations (Qiagen, Hilden, Germany). Gene transcription of *β-actin*, *HoxA7*, *HoxB8*, *HoxC8*, *HoxD8* was analyzed by a two-step RT-PCR: reverse transcription was performed with 2 μg of RNA (20 μL total volume; Ominscript RT Kit; Qiagen) according to the recommendations of the manufacturer. One microliter of cDNA was used as a template for the specific PCR reactions. Primers applied were *β-actin*-forward: 5'-TGACGGGGTCACCCACACTGTGCCCATCTA-3', *β-actin*-reverse: 5'-CTAGAAGCATTTGCGGTGGACGATGGAGGG-3' (661 bp fragment), *HoxA7*-forward: 5'-CCGCATGAAGTGGAAGAAAG-3', *HoxA7*-reverse: 5'-CAGTCCACAAAAGTTGGGAG-3' (347 bp fragment), *HoxB8*-forward: 5'-GCAATTTCTACGGCTACGAC-3' and *HoxB8*-reverse: 5'-GAAACAGAAGCTGGAGCGG-3' (434 bp fragment), *HoxC8*-forward: 5'-CACGTTCAAGACTTCTTCCACCACG-3' and *HoxC8*-reverse: 5'-GGTTCCAGAACCGAAGGATGAAGTG-3' (449 bp fragment), *HoxD8*-forward: 5'-ACAGCCGATTTTTACGACCC-3' and *HoxD8*-reverse: 5'-GCTTCCTTTTTCGTTTCCCC-3' (399 bp fragment).

For amplification, a DNA Engine PTC200 (MJ Research, Watertown, USA) thermocycler was used. Cycling conditions of the respective PCR were as follows: initial denaturation (4 min at 95℃), followed by the respective number of cycles (*β-actin*: 20; *HoxA7*: 29, *HoxB8*: 29, *HoxC8*: 29, *HoxD8*: 29) of denaturation (1 min at 94℃), annealing (1 min; *β-actin*: 57℃; *HoxA7*:

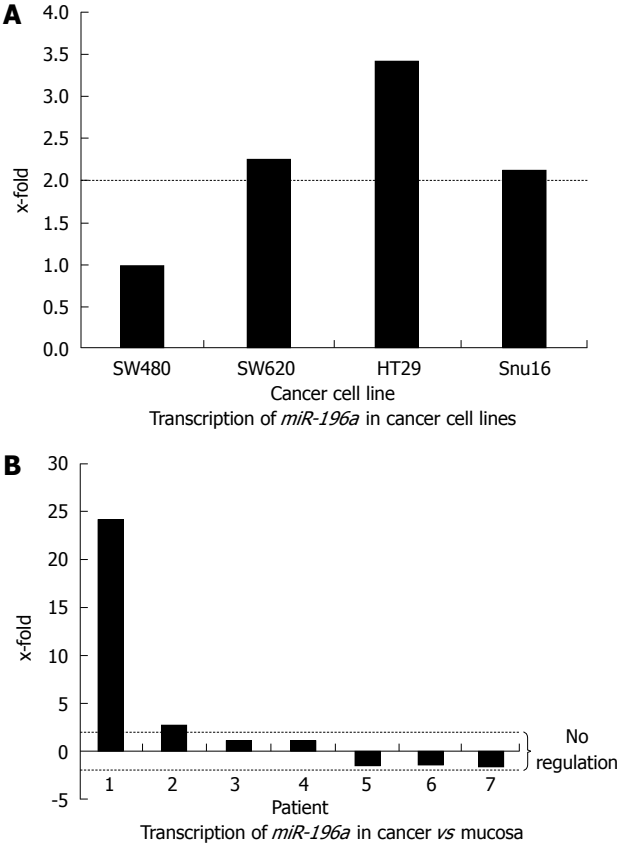

Figure 1 Transcription levels of *miR-196a* in cancer cell lines and human CRC. A: Cancer cell lines SW620, HT29 and Snu16 reveal increased *miR-196a* levels as compared to the primary colon cancer cell line SW480; B: *miR-196a* transcription is up-regulated in two of seven cancer samples in comparison to the matching mucosa sample. In contrast, no down-regulation in the respective tumor samples was observed.

58℃, *HoxB8*: 56℃, *HoxC8*: 62℃, *HoxD8*: 57℃) and elongation (2 min at 72℃). After the last cycle, a final extension (10 min at 72℃) was added and thereafter the samples were kept at 4℃. Seven microliters of the products were run on a 1.8% agarose gel, stained by ethidium bromide and analyzed under UV light.

Statistics analysis

The χ^2 test was used to compare all other patient and tumor characteristics by group. The *t* test was applied to compare results obtained from function assays. For all tests, $P < 0.05$ was considered significant.

RESULTS

miR-196a transcription in cancer cell lines

Real-time analyses of four cancer cell lines revealed U6 adjusted differences in regulation of *miR-196a* (Figure 1A). The SW480 cell line, which was initially isolated from a primary colon cancer, revealed the weakest transcription level. In contrast, SW620 cells, isolated from metastases of the same patient depicted a 2.25-fold up-regulation of *miR-196a*. HT-29, another colorectal cancer cell line revealed a 3.38-fold up-regulation of *miR-196a*. Similarly, SNU16 generated from metastases of a disseminated gastric cancer showed a 2.14-fold up-regulation of *miR-196a*.

miR-196a transcription in colon cancers versus mucosa

Real-time analyses of colon cancer and matching mucosa revealed an U6 adjusted up-regulation of *miR-196a* in two of seven colon cancers samples analyzed (24.3- and 2.5-fold, respectively; Figure 1B). In contrast, five of seven samples did not depict any transcription differences between tumor and mucosa (1.14-, 1.04-, -1.03-, -1.08- and -1.28-fold regulation, respectively).

Functional analysis using miR-196a transiently transfected SW480 cancer cells

Functional analyses did not depict any significant impact of *miR-196a* on proliferation (Figure 2A). Absorbance analyses after 4 d of cell culture revealed the following results: +m*iR-196a*: 1.506 ± 0.079, -m*iR-196a*: 1.533 ± 0.131; $P = 0.66$; (*vs* NS).

Interestingly, transfection with *miR-196a* decreased the adhesion of cancer cells to plastic and fibronectin but not to laminin (Figure 2B). Adhesion analyses revealed following results: for plastic surface: +m*iR-196a*: 10.2% ± 1.15%, -m*iR-196a*: 16.6% ± 1.73%; $P = 0.001$. For laminin coating: +m*iR-196a*: 3.86% ± 1.3%, -m*iR-196a*: 2.84% ± 0.95%; $P = 0.25$; (*vs* NS) and for fibronectin coating: +m*iR-196a*: 10.86% ± 1.64%, -m*iR-196a*: 13.8% ± 1.56%; $P = 0.08$; (NS).

In addition, *miR-196a* transfection resulted in a significant increase of migration and invasion (Figure 2C and D): Migration: +m*iR-196a*: 9.7% ± 3% *vs* -m*iR-196a*: 3.6% ± 2.4%; $P = 0.05$. Invasion: +m*iR-196a*: 12.6% ± 3% *vs* -*miR-196a*: 5.14% ± 3%; $P = 0.039$.

Influence of miR-196a on classical signal cascades

In order to analyze the relevance of *miR-196a* on activation of signal cascades we quantified phosphorylation of AKT and MEK (Figure 3A). Transient transfection with *miR-196a* resulted in an increased phosphorylation of (p)AKT but not of (p)MEK. These results imply that *miR-196a* increases activation of the *PI3K-AKT-mTor* signalling pathway.

Chemosensitivity analyses

Analyses of apoptosis did not reveal any significant impact of *miR-196a* (Figure 3B): +m*iR-196a*: 0.61% ± 0.08% *vs* -m*iR-196a*: 0.62% ± 0.07%, $P = 0.3$; (NS); nor in combination with 5-FU [+m*iR-196a*: 15.67% ± 1.45% *vs* -m*iR-196a*: 14.05% ± 0.74%, $P = 0.18$; (NS)] or irinotecan [+m*iR-196a*: 11.97% ± 0.51% *vs* -m*iR-196a*: 12.06% ± 1.36%, $P = 0.92$; (NS)]. However, *miR-196a* significantly increased chemosensitivity to oxaliplatin (+m*iR-196a*: 13.56% ± 2.08% *vs* -m*iR-196a*: 9.46% ± 1.19%, $P = 0.05$) and cisplatin (+m*iR-196a*: 23.11% ± 1.93% *vs* -m*iR-196a*: 18.42% ± 1.92%; $P = 0.04$). In summary, *miR-196a* increases chemosensitivity to platin derivates.

Lung metastases xenograft

Transient transfection of SW480 cancer cells with *miR-196a* resulted in a significant increase of pulmonary metastases growth after 7 wk of incubation: +m*iR-196a*: 7.5 ± 1.7 *vs* -*miR-196a*: 3.25 ± 0.96, $P = 0.009$ (Figure 3C).

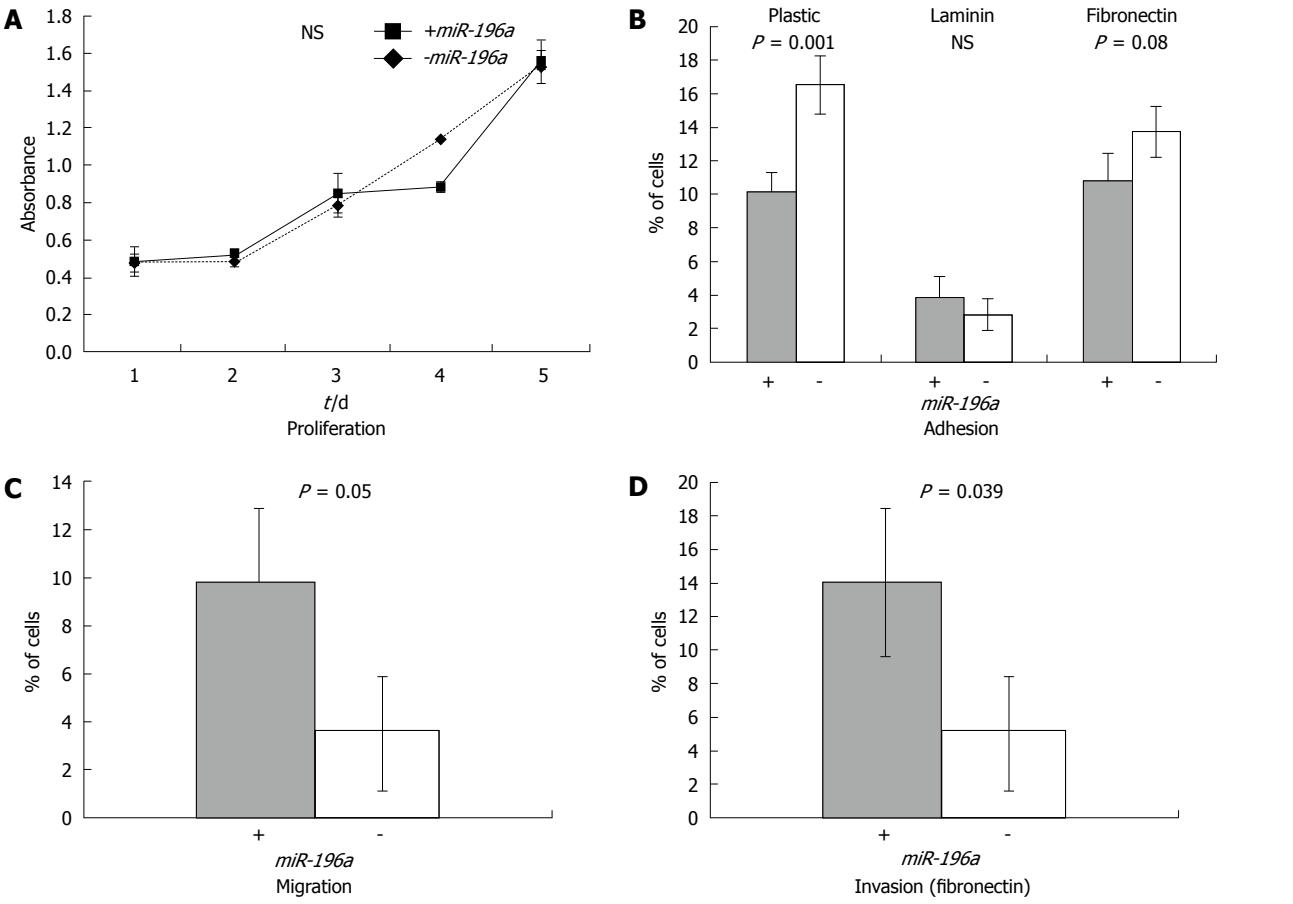

Figure 2 ***In vitro*** **effect of** ***miR-196a*** **in human CRC.** Transient *miR-196a* transfection significantly decreases adhesion, increases migration and invasion but does not impact on proliferation or apoptosis of SW480 colon cancer cells.

Verification of miR-196a target genes

Transient transfection of SW480 cells with *miR-196a* verified *HoxA7*, *HoxB8*, *HoxC8* and *HoxD8* as *miR-196a* targeted genes (Figure 4A). However, significant differences in target restriction were observed. While low *miR-196a* concentrations (20 nmol/L) sufficiently restricted *HoxB8* mRNA, higher concentrations were necessary to completely restrict *HoxC8* mRNA and to restrict a significant amount of *HoxD8* mRNA. However, the impact of *miR-196a* on *HoxD8* was weaker than on *HoxC8*. Only the highest *miR-196a* concentrations (240 nmol/L) decreased mRNA levels of *HoxA7*. These data verify the predicted *Hox* genes *HoxA7*, *HoxB8*, *HoxC8* and *HoxD8* as human targets of *miR-196a* but also reveal dose-dependent differences in restriction of target genes.

Dose-dependent inhibition of cellular adhesion

Transfection with *miR-196a* significantly decreased the adhesion of cancer cells to plastic in a dose-dependent manner. Numbers reflect the percentage of cells that adhered to the bottom of the well: 0 nmol/L *miR-196a*: 15.21% ± 0.47%; 40 nmol/L *miR-196a*: 14.27% ± 0.46%; $P = 0.07$; (NS); 80 nmol/L *miR-196a*: 12.43% ± 0.42%; $P = 0.002$ and 160 nmol/L *miR-196a*: 10.6% ± 0.3%; $P = 0.0003$ (Figure 4B).

DISCUSSION

Expression patterns of miRNAs are systematically altered in colon cancer as recently described by Schetter and colleagues[25]. In particular, Schetter *et al*[25] reported that at least 37 miRNAs are differentially expressed in colon cancer. Of those the expression profiles of *miR-20a*, *miR-21*, *miR-106a*, *miR-181b* and *miR-203* were validated. Interestingly, high *miR-21* expression was associated with poor survival.

We were interested in the relevance of *miR-196a* transcription for human colorectal cancer progression for specific reasons. Yekta and colleagues described *HoxB8* as a restriction target of *miR-196a* and predicted *HoxA7*, *HoxC8* and *HoxD8* as additional restriction targets in humans[22]. *Hox* genes are known to be master regulators of embryogenesis and oncogenesis[15]. We were able to confirm these data presented by Yekta and colleagues, as mRNA levels of those four *Hox* genes were reduced by *miR-196a*. However, dose-dependent differences in target restriction were observed. While low *miR-196a* concentrations resulted in a complete restriction of *HoxB8* mRNA, higher concentrations of *miR-196a* were mandatory to completely restrict *HoxC8* mRNA and to significantly decrease *HoxD8* mRNA levels. In contrast, even the highest *miR-196a* concentrations did not result in a complete restriction

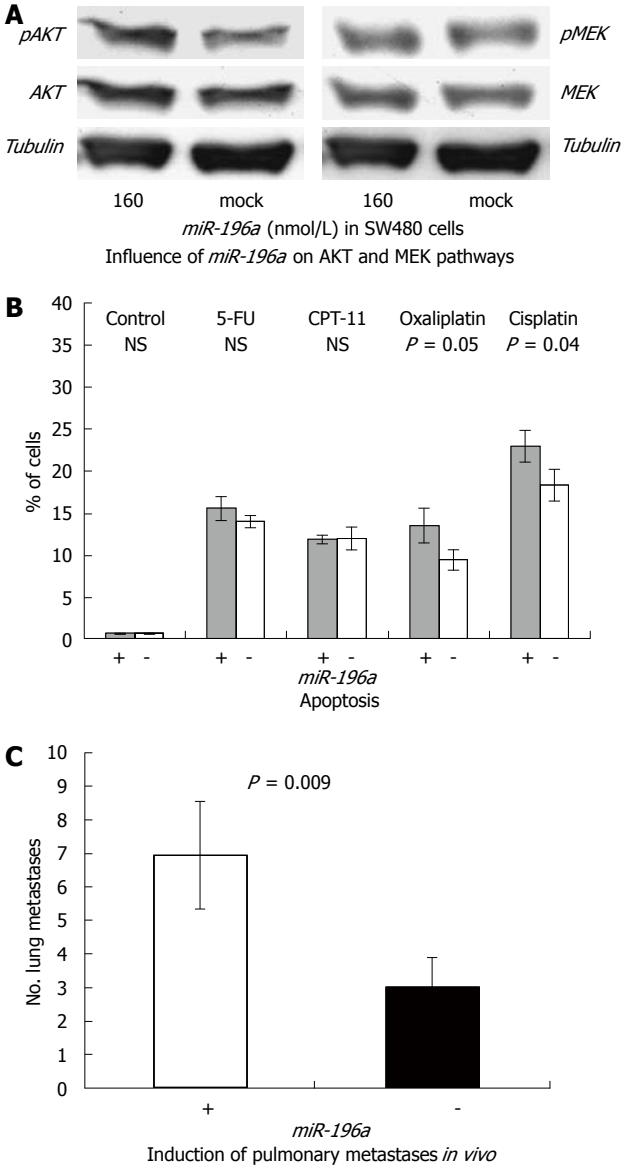

Figure 3 Impact of *miR-196a* on cellular signaling, *in vitro* chemosensitivity and *in vivo* induction of lung metastases. A: *miR-196a* transfection activates the AKT pathway but does not impact on the MEK pathway; B: *miR-196a* transfection significantly increases chemosensitivity towards oxaliplatin and cisplatin but not towards 5-FU or irinotecan; C: *miR-196a* significantly promoted growth of lung metastases in a xenograft biosystem after tail-vein injection and 7 wk of incubation.

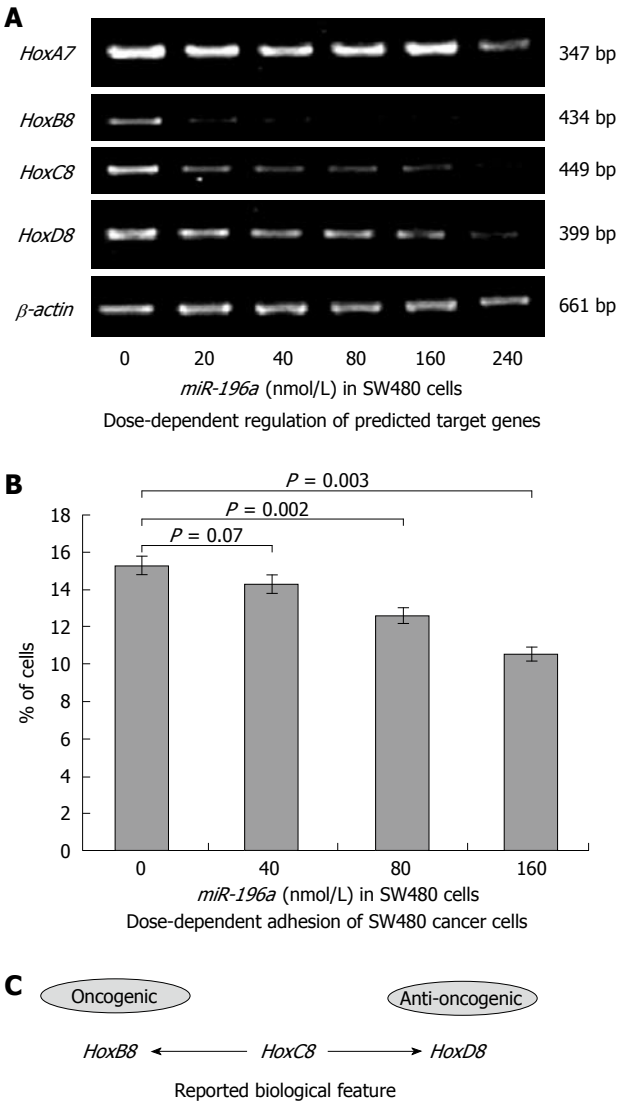

Figure 4 Dose-dependence of *miR-196a* promoted effects. A: *miR-196a* decreases *HoxA7*, *HoxB8*, *HoxC8* and *HoxD8* mRNA levels with a dose-dependent and gene-specific character; B: *miR-196a* inhibits cancer cell adhesion to plastic covers in a dose-dependent manner; C: Biological features of *HoxB8*, *HoxC8* and *HoxD8* as reported in literature. *HoxB8* exerts an oncogenic effect, while *HoxD8* might have tumor-suppressive relevance. For *HoxC8* both, pro-oncogenic and anti-oncogenic features, have been reported.

of *HoxA7*. These data clearly reveal mRNA specific and dose-dependent target restriction. To clarify the dose-dependence of *miR-196a* we performed adhesion assays after transfection with different concentrations of *miR-196a*. These assays revealed a dose-dependent inhibition of tumor cell adhesion.

To further analyze the impact of *miR-196a* on tumor cells, we then performed functional assays and found that high *miR-196a* concentrations increased migration and invasion of cancer cells in trans-well assays and inhibited adhesion to different surfaces and matrix proteins. Chemosensitivity assays with standard chemotherapeutics revealed that *miR-196a* does not sensitise against 5-FU nor irinotecan, but does sensitize against the platin derivatives oxaliplatin and cisplatin. However, *miR-196a* did not impact on proliferation or apoptosis of colon cancer cells.

Analyzing signaling cascades that are often altered in human cancer, we observed that induction of the pro-migratory phenotype is most likely linked to activation of the *PI3K-AKT-mTor* pathway, as *miR-196a* increased the level of pAKT. In contrast, no change in the pMEK/MEK ratio was observed. Our data are consistent with earlier reports showing that overexpressed miRNAs can act as oncogenes. A well known example is *miR-17-92*, which is significantly increased in small-cell lung cancer and correlates with a poor prognosis[20]. Interestingly, the known targets of the *miR-17-92* include the two tumor suppressor genes *PTEN* and *RB2*[21]. As a consequence, restriction of *PTEN* unleashes the *PI3K-AKT-mTor* pathway as also observed for *miR-196a*. However, the exact mode of action of *miR-196a* has still to be analyzed.

Quantitative real-time PCR of *miR-196a* in matching colon cancer and colon mucosa samples showed an up-regulation in 28% of samples. In contrast, all other cancer samples revealed no regulation at all. Most interestingly, the metastatic cancer cell lines SW620 and HT29 showed a significant up-regulation of *miR-196a* in contrast to SW480 cells isolated from a primary colon cancer. Therefore, *miR-196a* is up-regulated in a subset of colorectal cancers and might exert an oncogenic function, when transcribed at a high level. Matching these observations, Croce and colleagues recently found that 75% of pancreatic cancers expressed *miR-196a* at a high level, predicting poor patient survival (14.3 mo *vs* 26.5 mo) when investigating the miRNA transcription pattern in pancreatic adenocarcinoma with large scale miRNA chips[24]. Therefore, similar mechanisms seem possible for pancreatic and colorectal cancer.

To verify the oncogenic potential of high *miR-196a* concentrations, we further analyzed the impact of *miR-196a* in an *in vivo* lung metastases xenograft biosystem. After transient transfection of cells with high concentrations of *miR-196a* prior to tail-vein injection, mice developed significantly more pulmonary metastases within 7 wk as compared to mock-transfected cells.

In summary, we observed an oncogenic effect of high *miR-196a* concentrations. However, several data imply that *miR-196a* might function as a double-edged sword with opposing effects at different concentration for following reasons. (1) *miR-196a* is transcribed in colon mucosa at low levels, implying a role for the epithelial phenotype. (2) A hypothesized suppressive effect of low *miR-196a* transcription levels on tumor dissemination might be exerted through a dose-dependent restriction of *miR-196a* target genes *HoxB8*, *HoxC8* and *HoxD8*. Up-regulation of *HoxC8* and *HoxB8* in colorectal cancer was reported as early as 1997, however the relevance of those genes for carcinogenesis had not been analyzed[26]. A relevant leukemogenic property of *HoxB8* mediated through inhibition of differentiation has been described for acute myeloid leukemia[27,28]. These data are intriguing, as low concentrations of *miR-196a* completely restrict *HoxB8*, thus erasing the pro-oncogenic and leukemogenic effects of *HoxB8*. (3) Only very limited data concerning the relevance of *HoxD8* is available, indicating that *HoxD8* are up-regulated after chemical induced re-differentiation of neuroblastoma cells[29]. However, this observation is of particular interest, as high *miR-196a* concentrations are needed to significantly reduce *HoxD8* mRNA levels, which might result in an inhibition of differentiation, thus promoting oncogenic features as observed in our analyses. (4) The data concerning the relevance of *HoxC8* is unclear. Both pro- and anti-oncogenic influences have been discussed. In particular, *HoxC8* was reported to be a retinoic acid induced gene, rescuing *APC* mutants in zebrafish[30]. In contrast, studies on prostate cancer have reported a correlation with aberrant *HoxC8* expression and a malignant phenotype[31,32]. As *Hox* genes are master transcription factors, they might exert different functions at variable expression levels. However, the observation of Croce and colleagues that *miR-196a* predicts poor survival in pancreatic cancer might rather correlate with inhibition of *HoxD8* than *HoxB8* expression, as *HoxD8* has a suppressive and *HoxB8* a progressive character in the literature[24]. Further studies analyzing the clinical and biological impact of *miR-196a*, as well as additional large scale analyses of restriction targets, are warranted.

COMMENTS

Background

MicroRNAs (miRNAs) are small RNA molecules regulating gene expression in vertebrae and non-vertebrae. In humans, more than 50% of all known miRNA genes are located in cancer-associated regions, indicating that miRNAs might play an important role in oncogenesis. Some miRNAs are known to function as oncogenes, while others act as tumor suppressors inhibiting tumor growth.

Research frontiers

Hox proteins are major transcription factors that play a crucial role during embryogenesis, organogenesis and oncogenesis. The miRNA *miR-196a* depicts complementarity to the mRNA of *HoxB8*, *HoxC8* and *HoxD8*. Therefore, the relevance of *miR-196a* for human tumorigenesis has been discussed.

Innovations and breakthroughs

High levels of *miR-196a* activated oncogenic pathways inside the human tumor cells and induced tumor cell detachment, migration and invasion. In addition, *miR-196a* promoted growth of lung metastases in mice. However, *miR-196a* also increased the chemosensitivity towards platin derivatives such as cisplatin and oxaliplatin.

Applications

High levels of *miR-196a* might predict response of cisplatin- or oxaliplatin-containing chemotherapies. In future, suppression of *miR-196a* by anti-miR technologies might inhibit tumor progression and dissemination.

Terminology

miRNAs are endogenous small RNA molecules of 20-25 nucleotides length, regulating gene expression by inhibiting transcription, inducing direct cleavage of the targeted mRNAs or blocking translation through their complementarity *versus* targeted mRNAs at 3' untranslated regions.

Peer review

This is a very interesting study which contributes to our understanding of colorectal cancer, its development and prognosis. The paper is well written.

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Bile-acid-activated farnesoid X receptor regulates hydrogen sulfide production and hepatic microcirculation

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Abstract

AIM: To investigate whether the farnesoid X receptor (FXR) regulates expression of liver cystathionase (CSE), a gene involved in hydrogen sulfide (H_2S) generation.

METHODS: The regulation of CSE expression in response to FXR ligands was evaluated in HepG2 cells and in wild-type and FXR null mice treated with 6-ethyl chenodeoxycholic acid (6E-CDCA), a synthetic FXR ligand. The analysis demonstrated an FXR responsive element in the 5'-flanking region of the human *CSE* gene. The function of this site was investigated by luciferase reporter assays, chromatin immunoprecipitation and electrophoretic mobility shift assays. Livers obtained from rats treated with carbon tetrachloride alone, or in combination with 6-ethyl chenodeoxycholic acid, were studied for hydrogen sulphide generation and portal pressure measurement.

RESULTS: Liver expression of CSE is regulated by bile acids by means of an FXR-mediated mechanism. Western blotting, qualitative and quantitative polymerase chain reaction, as well as immunohistochemical analysis, showed that expression of CSE in HepG2 cells and in mice is induced by treatment with an FXR ligand. Administration of 6E-CDCA to carbon tetrachloride treated rats protected against the down-regulation of CSE expression, increased H_2S generation, reduced portal pressure and attenuated the endothelial dysfunction of isolated and perfused cirrhotic rat livers.

CONCLUSION: These results demonstrate that CSE is an FXR-regulated gene and provide a new molecular explanation for the pathophysiology of portal hypertension.

Key words: Nuclear receptor; Farnesoid X receptor; Cystathionase; Hydrogen sulfide; Portal hypertension

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INTRODUCTION

In mammals, cysteine is provided through the diet or by the trans-sulfuration pathway, in which L-cysteine is synthesized by sulfur transfer from L-methionine to L-serine. Cystathionine-γ-lyase (CSE) is a pyridoxal 5'-phosphate-dependent enzyme, which catalyze the final essential step of the trans-sulfuration pathway; the conversion of L-cystathionine into L-cysteine, α-ketobutyrate and ammonia[1-3]. Cysteine is further irreversibly metabolized in the liver to yield glutathione[4-6], taurine[7] and hydrogen sulfide (H_2S), a gaseous bioactive molecule[3,8]. CSE is the main enzyme involved in H_2S generation by vascular smooth muscle cells[9,10] and accounts for the vasodilatory effect of H_2S in the systemic circulation[11,12]. In the liver, H_2S generated by hepatocytes and hepatic stellate cells exerts vasodilatory activities and reduces intrahepatic resistance counter-acting the effect of vasomotor mediators on presinusoidal myofibroblasts[13,14].

An alteration of the trans-sulfuration pathway is common in chronic liver diseases, with hyperhomocysteinemia occurring in two-thirds of cirrhotic

patients, regardless the etiology of liver damage[15,16]. An imbalance of the trans-sulfuration pathway linked to reduced expression and activity of CSE is observed in rodent models of liver injury. This alteration leads to a combination of hyper-homocysteinemia and reduced generation of H_2S, translating into an enhanced vasomotor tone and increased intrahepatic resistance[17,18]. Homocysteine is a negative regulator of nitric oxide (NO) bioactivity in endothelial cells. Perfusion of the normal and cirrhotic rat livers with homocysteine results in attenuated NO generation and impaired hepatic vasodilation in response to acetylcholine and shear stress, highlighting the critical role of intermediates of the trans-sulfuration pathway in regulating intrahepatic vasomotor activity[18].

Little is known about the mechanism responsible for the reduced expression of CSE in the injured liver. The fact that CSE expression is modulated during development, being detected at very low levels in embryos while a gradual increase of expression occurs after birth, suggests that genes involved in liver differentiation or proliferation might control the expression of this gene[1].

The farnesoid X receptor (FXR, NR1H4), a member of the ligand-activated nuclear hormone receptor superfamily, is primarily expressed in the liver, kidney, and intestine[19]. It functions as a heterodimer with the retinoid X receptor (RXR)[20] and binds to response elements in the promoters of target genes involved in bile acid homeostasis, and lipid and glucose metabolism[21]. The FXR-RXR heterodimer binds with highest affinity to an inverted repeat sequence in which consensus receptor-binding hexamers are separated by one nucleotide (IR1: AGGTCA*g*TGACCT)[22]. FXR functions as a bile acid sensor, and upon activation, it reduces the conversion of cholesterol into bile acids and increase bile acid excretion from hepatocytes by activating canalicular transporters. In the present study, we investigated whether FXR regulates H_2S generation. Our results demonstrate that the 5'-flanking region of the human *CSE* gene contains an FXR response element (AGTTCA*g*TGTACCT) and that FXR activation *in vitro* and *in vivo* enhances CSE expression and activity, and directly stimulates H_2S generation. These data suggest that FXR directly regulates the generation of a vasodilatory mediator in the liver and provide new pathophysiological insights into the molecular mechanism of portal hypertension.

MATERIALS AND METHODS

Cell culture

HepG2 cells were grown at 37℃ in Minimum Essential Medium with Earl's salts containing 10% fetal bovine serum (FBS), 1% L-glutamine and 1% penicillin/streptomycin. Cells were serum starved for 24 h and then stimulated with 6E-CDCA (6-ethyl-chenodexycholic acid) 10 μmol/L for 18 h. At the end of treatment, total RNA and proteins were extracted to investigate the expression of CSE. Cells were also fixed in acetone and stained with a CSE monoclonal antibody (provided by Dr. N. Nishi, Kagawa Medical School, Japan)[19].

RNA extraction

Total RNA was isolated from liver or HepG2 cells using the TRIzol reagent according to the manufacturer's specifications (Invitrogen, Milan, Italy). One microgram of RNA was purified from genomic DNA by DNase- I treatment (Invitrogen) and reverse-transcribed using random hexamer primers with Superscript Ⅱ (Invitrogen) in a 20-μL reaction volume.

Qualitative and quantitative real-time polymerase chain reaction (RT-PCR)

The amplification of cDNA (50 ng) was achieved in a 50-μL mixture containing 200 nmol/L dNTPs, 1.5 mmol/L $MgCl_2$, 200 nmol/L gene-specific sense and antisense primers and 1 U Platinum *Taq* DNA Polymerase (Invitrogen). All PCR primers were designed using software PRIMER3-OUTPUT using published sequence data from the NCBI database (Table 1). Quantitative RT-PCR conditions were as described previously[13].

Western blotting anti-CSE

Total lysates were prepared by solubilization of cells or liver homogenates in NuPage sample buffer (Invitrogen) containing Sample Reducing Agent (Invitrogen) and separated by PAGE. The proteins were then transferred to nitrocellulose membranes (Bio-Rad) and probed with primary antibodies CSE[17,23] and tubulin (Sigma). The anti-immunoglobulin G horseradish peroxidase conjugate (Bio-Rad) was used as the secondary antibody, and specific protein bands were visualized using Super Signal West Dura (Pierce), following the manufacturer's suggested protocol.

Immunohistochemical analysis of CSE

Immunohistochemical analysis of CSE was performed in HepG2 cells and in liver sections from FXR +/+ and FXR -/- mice not treated and treated with CCl_4. Cells were fixed in 95% acetone for 5 min and endogenous peroxidase was blocked using Dako Peroxide Blocking (DAKO) for 10 min. An anti-CSE monoclonal antibody[23] was used at a dilution of 1:100 for 1 h at room temperature and a biotin-streptavidin-HRP detection/DAB substrate chromogen system was used to visualize the detected proteins. For liver staining, portions of the right and left liver lobes (15 mg/each) from each animal were fixed in 10% formalin, embedded in paraffin, sectioned, blocked with Dako Peroxide Blocking and stained with CSE monoclonal antibody diluted 1:100 for 1 h at room temperature. A biotin-streptavidin-HRP detection system was used using DAB substrate as the chromogen.

Measurement of CSE activity

The CSE activity was assessed accordingly to the method reported by Ogasawara *et al*[24] with minor modifications;

Table 1 Primers used for quantitative and qualitative PCR

Gene	Forward	Reverse
hGAPDH	GAAGGTGAAGGTCGGAGT	CATGGGTGGAATCATATTGGAA
hCSE	CACTGTCCACCACGTTCAAG	GTGGCTGCTAAACCTGAAGC
hCSE-IR1	CATTACAGAGTTCAGTGTACCT	GCAGCTTGGATTCTCATCAGTC
r18S	GCAATTATTCCCCATGAACG	GGCCTCACTAAACCATCCAA
rCSE	GTATTGAGGCACCAACAGGT	GTTGGGTTTGTGGGTGTTTC
rFXR	TGGACTCATACAGCAAACAGAGA	GTCTGAAACCCTGGAAGTCTTTT
rαSMA	GCTCCATCCTGGCTTCTCTA	TAGAAGCATTTGCGGTGGAC
rCOL1α1	TGCTGCCTTTTCTGTTCCTT	GGATTTGAAGGTGCTGGGTA
rSHP	CCTGGAGCAGCCCTCGTCTCAG	AACACTGTATGCAAACCGAGGA
m18S	ACCGCAGCTAGGAATAATGGA	GCCTCAGTTCCGAAAACCA
mCSE	TGCTGCCACCATTACGATTA	GATGCCACCCTCCTGAAGTA
mα1-collagen	ACGTCCTGGTGAAGTTGGTC	CAGGGAAGCCTCTTTCTCCT

h: Human; m: Mouse; r: Rat; hCSE-IR1: Primers used for real-time PCR of the CSE promoter in chromatin immunoprecipitation assay.

DL-propargylglycine (final 1 mmol/L) instead of 4,4-dithiodipyridine (final 3 mmol/L) was used to inactivate CSE. This method utilizes colorimetry for the determination of pyruvate produced from β-chloro-L-alanine by a CSE-catalyzed elimination reaction, coupling a color-generating enzymatic reaction with pyruvate oxidase and peroxidase. The CSE-specific activity was expressed as the ratio (between sample and sample blank) of absorbance at 727 nm per microgram of protein per seconds of incubation. Sulfide concentrations and production from liver supernatants were measured as previously described[13].

Transactivation assay

For the luciferase assay, 24 h before transfection, 10×10^5 HepG2 cells were plated in six-well plates and cultured in E-MEM supplemented with 1% penicillin/streptomycin, 1% L-glutamine and 10% FBS. Cells were grown at 37℃ in 5% CO_2. All the transfections were made using Fugene HD according to manufacturer's specifications (Roche) and performed using 1 μg pGL3 or pGL3 $(CSE\text{-}IR1)_{4X}$ or $pGL3CSEIR1_{mutated}$ as reporter vectors, 200 ng pCMV-βgalactosidase as an internal control for transfection efficiency, and 100 ng of each expression plasmid pSG5-FXR and pSG5-RXR. The pGEM vector was added to normalize the amounts of DNA transfected in each assay to 2.5 μg/well. Forty-eight hours post-transfection, HepG2 cells were stimulated with a dose response of 6E-CDCA (from 0.01 to 10 μmol/L) or with bile acids (25 μmol/L) for 18 h. Control cultures received vehicle (0.1% DMSO) alone. For the competition assay, an FXR antagonist, such as guggulsterone, was used at 50 μmol/L alone, or in combination with 6E-CDCA 10 μmol/L, for 18 h. Cells were lysed in 100 μL diluted reporter lysis buffer (Promega), and 5 μL of cellular lysate was assayed for luciferase activity using Luciferase Assay System (Promega). Luminescence was measured using an automated luminometer. Luciferase activities were normalized for transfection efficiencies by dividing the relative light units by β-galactosidase activity. All experiments were done in triplicate and were repeated at least once.

Electrophoretic mobility shift assay (EMSA)

Preparation of nuclear extract from HepG2 cells was done using NE-PER (Pierce). The probes used for EMSA (CSERE-IR1, $CSERE\text{-}IR1_{mutated}$ and FXRE-IR1) were labeled with biotin using Biotin 3' end DNA labelling kit (Pierce) according to the manufacturer's instructions. For EMSA, 5 μg of nuclear extract from HepG2 cells not treated or stimulated with 6E-CDCA 10 μmol/L were incubated with 15 fmol of the CSERE-IR1 probe, while 5 μg of nuclear extract from HepG2 stimulated with 6E-CDCA was incubated with $CSERE\text{-}IR1_{mutated}$ and FXRE-IR1 probes in a total volume of 20 μL of binding buffer (50 mmol/L NaCl, 10 mmol/L Tris-HCl, pH 7.9, 0.5 mmol/L EDTA, 10% glycerol, 1 μg of poly dI-dC) for 20 min at room temperature. For competition assays, an excess of CSERE-IR1 unlabeled oligonucleotides were pre-incubated with nuclear extract from 6E-CDCA-treated cells for 15 min prior to the addition of the biotin-labeled CSERE-IR1 probe. For antibody-mediated supershift assay, extracts from stimulated cells were pre-incubated with 1 μg anti-FXR antibody H-130 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or with 1 μg anti-RXR antibody ΔN 197 (Santa Cruz Biotechnology) at room temperature for 20 min before the addition of the biotin-labeled CSERE-IR1 probe. The reactions were loaded on a 6% polyacrylamide non-denaturing gel in 0.5 × Tris-borate-EDTA buffer and electrophoresed for 1 h at 100 V. The protein/DNA complexes were then transferred to positively charged nylon membrane (Pierce) and the supershift was detected using the Chemiluminescent Nucleic Acid Detection Module (Pierce).

Chromatin immunoprecipitation (ChIP)

A ChIP assay was performed according to the manufacturer's protocols (Abcam Ltd, Cambridge, UK) with minor modifications. In brief, HepG2 cells serum starved for 24 h, not treated or stimulated with 6E-CDCA 10 μmol/L for 18 h, were cross-linked with 1% formaldehyde at room temperature, and then the reaction was terminated by the addition of glycine to a final concentration of 0.125 mol/L. Cells were washed in ice-cold PBS and lysed with SDS lysis buffer (1% SDS, 10 mmol/L EDTA, and 50 mmol/L Tris-HCl, pH 8). Cellular lysates were diluted with ChIP dilution buffer, sonicated, and immunoprecipitated with specific

antibodies: anti-FXR or anti-CD4 as a negative control (Santa Cruz Biotechnology). Immunoprecipitates were collected with protein A beads (Amersham Bioscience) and washed sequentially, first with a low-salt wash buffer and then with high-salt wash buffer using the manufacturer's recommended procedures. DNA was eluted by addition of 1% SDS and 0.1 mol/L $NaHCO_3$, and the cross-linking reactions were reversed by heating the mixture to 65℃ overnight. The DNA was recovered from immunoprecipitated material by proteinase K treatment at 65℃ for 1 h followed by phenol/chloroform (1:1) extraction, ethanol precipitation and dissolved into 50 μL of water. Five microliters was used for quantitative real-time PCR. Five microliters of PCR reactions were extracted after 40 complete cycles for visualization on agarose gels and stained with ethidium bromide.

In vivo experimental studies

All animal procedures were approved by the Animal Study Committees of the University of Perugia. In the first study, the effect of FXR ligands on liver expression of CSE was investigated in FXR +/+ and FXR -/- mice treated by intraperitoneal injection of 6E-CDCA 5 mg/kg body weight for 3 d while control animals were treated with vehicle alone (methyl-cellulose). C57BL/6j mice, obtained from Charles River Breeding Laboratories (Monza, Italy), and homozygous C57BL/6j FXR -/- mice, obtained from Gonzalez *et al*[25] were used with a 12 h light/12 h dark cycle with free access to water and standard laboratory chow diet. At the end of the study, mice were sacrificed and their livers were removed to measure the relative mRNA expression of CSE, the activity of the enzyme and the production of H_2S. In the second study, cirrhosis was induced in FXR +/+ and FXR -/- mice by administering phenobarbital sodium (35 mg/dL) to the mice with drinking water for 3 d, followed by intraperitoneal injection of 100 μL/100 g body weight of CCl_4 in an equal volume of paraffin oil twice 1 wk for 6 wk. CCl_4 administered mice were treated with intraperitoneal injection of 6E-CDCA 5 mg/kg body weight, while control animals were treated with vehicle alone (methyl-cellulose). Mice were sacrificed and their livers were removed for histological, histochemical, and real-time PCR analysis. Blood samples were taken for biochemical analysis. In the third study, cirrhosis was induced in rats obtained from Harlan Nossan (Italy) by administering phenobarbital sodium (35 mg/dL) with drinking water for 3 d, followed by intraperitoneal injection of 100 μL/100 g body weight of CCl_4 in an equal volume of paraffin oil twice 1 wk for 6 wk. After the treatment with CCl_4, animals were administered with an intraperitoneal injection of 6E-CDCA, 10 mg/kg for 5 d while control animals were treated with vehicle alone (methyl-cellulose). At the end of the treatment, analysis of hepatic vascular responses to norepinephrine (from 10 nmol/L to 10 μmol/L) was performed using the isolated perfused rat liver preparation[26]. Briefly, a median laparotomy was performed and an PE-50 catheter was introduced into the inferior mesenteric vein and advanced to the portal vein for the measurement of portal pressure. The liver was perfused in a recirculating mode with Krebs solution equilibrated with CO_2, using a peristaltic pump as previously described[27]. The perfusion pressure was continuously monitored and recorded with a strain-gauge transducer connected to a PowerLab PC (A.D. Instruments, Milford, MA, USA). The preparation was allowed to stabilize for 20 min. The global viability of livers was assessed by standard criteria: gross appearance, stable pH of the perfusate, stable perfusion pressure for 20 min, and bile flow of > 1 μL/min per gram liver. The flow rate during each individual perfusion was maintained at a constant rate of 20 mL/min. Two additional groups of normal and cirrhosis rats were sacrificed and liver specimens were snap frozen in liquid nitrogen and stored at -70℃.

Serum biochemistry analysis

Serum bilirubin, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured by routine clinical chemistry testing performed on a Hitachi 717 automatic analyzer.

Liver histology

For histological examination, portions of the right and left liver lobes were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with Sirius red.

Western blotting anti-smooth muscle actin (αSMA)

Total cellular proteins of frozen tissues were extracted using Tissue Protein Extraction reagent (Pierce) and solubilized in NuPage sample buffer (Invitrogen) containing Sample Reducing Agent (Invitrogen). Proteins were resolved by electrophoresis on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad). After protein transfer, filters were probed with an αSMA primary antibody (Santa Cruz Biotechnology) for 1 h at room temperature. The anti-immunoglobulin G horseradish peroxidase conjugate (Bio-Rad) was used as the secondary antibody, and specific protein bands were visualized using Super Signal West Dura (Pierce), following the manufacturer's suggested protocol.

Statistics analysis

All values are expressed as mean ± SE of *n* observations per group. Comparisons of more than two groups were made with a one-way ANOVA with post-hoc Tukey's test. Comparison of two groups was made using Student's *t* test for unpaired data when appropriate. Differences were considered statistically significant if P was < 0.05.

RESULTS

CSE expression is regulated by FXR activation *in vitro*

We first investigated whether FXR activation modulates *CSE* gene expression. Serum-starved HepG2 cells, wild-type and stimulated with 10 μmol/L 6E-CDCA (a synthetic FXR ligand that activates FXR with an EC_{50} of about 300 nmol/L) were used in these experiments. As illustrated in Figure 1, FXR activation by this agent resulted in a robust induction of CSE expression

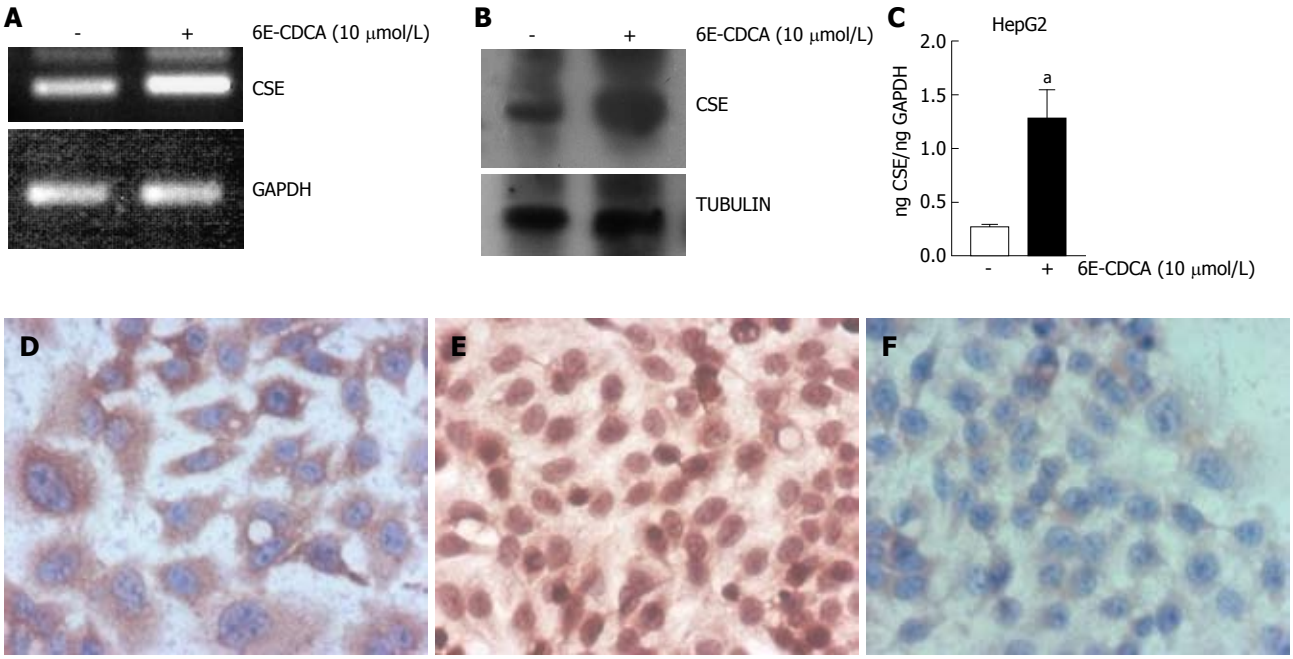

Figure 1 ***CSE*** **gene expression is regulated by bile acids.** A, B: Qualitative and quantitative PCR showing the up-regulation of CSE mRNA in HepG2 cell line stimulated with FXR ligand 6E-CDCA (10 μmol/L) for 18 h. Data are shown as mean ± SD of three experiments. $^{a}P < 0.05$ *versus* not stimulated cells; C: Western blotting analysis showing the up-regulation of CSE protein in HepG2 cell line stimulated by 6E-CDCA 10 μmol/L for 18 h; D, E: Immunohistochemistry analysis of CSE expression in HepG2 cells non-treated (D) and treated (E) with 6E-CDCA (10 μmol/L) for 18 h (Magnification × 40); F: Negative control was obtained by cell staining only with the secondary antibody.

as measured by qualitative and quantitative PCR (Figure 1A and B; $n = 3$, $P < 0.05$ *vs* not stimulated cells) and Western blotting analysis (Figure 1C). Consistent with these findings, the immunohistochemical analysis of CSE expression demonstrated a significant increase in cell expression of this protein in HepG2 cells exposed to 10 μmol/L 6E-CDCA for 18 h (Figure 1E). These data establish that FXR activation in hepatocytes up-regulates CSE mRNA and protein expression.

Identification of an IR-1 sequence in the human CSE promoter

Having showed that the expression of human *CSE* gene is induced in response to FXR activation, we then investigated whether the CSE promoter contains any potential FXR binding sites. FXR binds preferentially to the IR1 element, and a putative IR1 sequence (CSE-IR1: AGTTCA*g*TGTACCT) was identified in the 5'-flanking region of the *CSE* gene (Figure 2A). This sequence is located 699 base pairs upstream of the transcriptional start site. To explore the functional role of this non-canonical IR1 sequence, four copies of the CSE-IR1 were cloned in the pGL3 basic vector [pGL3 $(CSE\text{-}IR1)_{4X}$]. Additionally (Figure 2B), a construct containing a mutated IR1 site ($CSE\text{-}IR1_{mutated}$: ATTTCT*g*TGTACCT) was generated and cloned in the pGL3 vector ($pGL3CSE\text{-}IR1_{mutated}$). Using these reagents we investigated whether the identified FXR response element confers responsiveness to bile acid stimulation on the luciferase reporter gene. For this purpose, HepG2 cells co-transfected with pSG5-FXR and pSG5-RXR expression vectors were transiently transfected with the pGL3 $(CSE\text{-}IR1)_{4X}$ and then treated with natural FXR ligands: deoxycholic acid (DCA), litocholic acid (LCA), cholic acid (CA), chenodeoxycholic acid (CDCA) and the synthetic FXR ligand 6E-CDCA at 25 μmol/L for 18 h. As show in Figure 2C, treating HepG2 cells with natural FXR ligands resulted in an approximately two to three-fold increase in luciferase activity, while the treatment with synthetic ligand resulted in an approximately eight-fold increase in luciferase activity ($n = 3$, $P < 0.05$ *vs* not treated cells). 6E-CDCA-mediated induction of reporter gene expression was concentration-dependent with an EC50 of 300 nmol/L (Figure 2D; $n = 3$, $P < 0.05$ *vs* not treated cells).

To further confirm the role of CSE-IR1 in mediating CSE induction in response to FXR activation, HepG2 cells co-transfected with pSG5-FXR and pSG5-RXR expression vectors were then transfected with pGL3 or pGL3 $(CSE\text{-}IR1)_{4X}$ or $pGL3CSE\text{-}IR1_{mutated}$ and then stimulated with 6E-CDCA 10 μmol/L for 18 h. Cells transfected with the pGL3 basic vector alone were used as an internal control (Figure 2E columns 1 and 2). As expected, co-transfection of pSG5-FXR and pSG5-RXR with pGL3 $(CSE\text{-}IR1)_{4X}$ resulted in a substantial increase in luciferase activity compared to co-transfection with the luciferase reporter vector alone. (Figure 2E, columns 1 and 3; $n = 3$, $P < 0.05$ *vs* not stimulated pGL3 transfected cells). The construct containing the wild-type IR-1 [pGL3 $(CSE\text{-}IR1)_{4X}$] was found to cause about a four-fold increase in luciferase expression in the presence of a synthetic FXR ligand [Figure 2E, columns 3 and 4; $n = 3$, $P < 0.05$ *vs* not stimulated pGL3 $(CSE\text{-}IR1)_{4X}$ transfected cells]. The transactivation was abolished in cells transfected with a reporter gene in which the IR1 sequence was mutated [Figure 2E, column 5; $n = 3$, $P < 0.05$ *vs* not stimulated pGL3 $(CSE\text{-}IR1)_{4X}$ transfected cells] and the luciferase activity of the $pGL3CSE\text{-}IR1_{mutated}$ was similar to pGL3 basic. Similar results were obtained

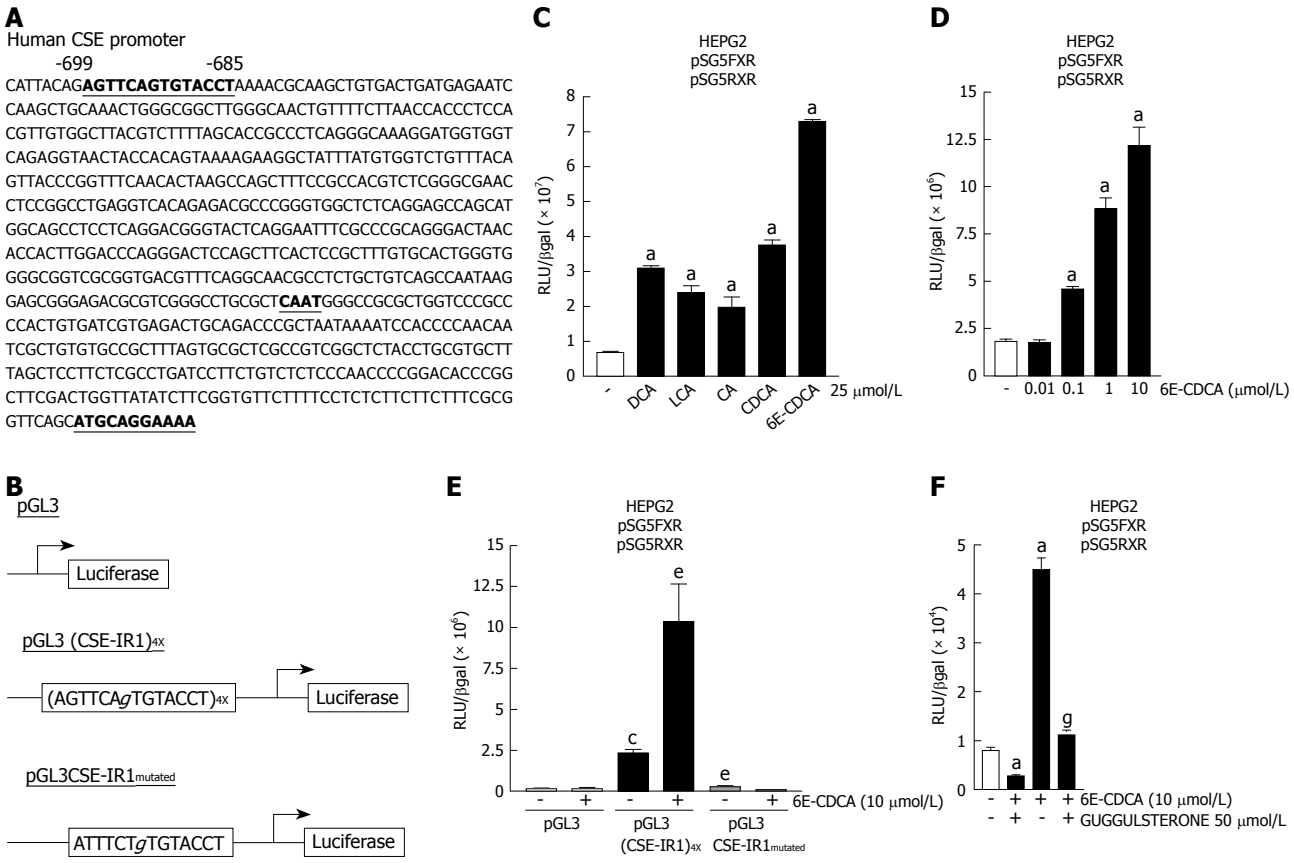

Figure 2 An FXR responsive element is expressed in the CSE promoter. A: Analysis of the promoter of the human *CSE* gene, showing a putative IR-1 site at -699/-685 base pairs upstream of the transcriptional start site ATG; B: Schematic representation of reporter constructs containing four CSE-IR1 elements [pGL3 (CSE-IR1)$_{4x}$] or the mutated CSE-IR1 (pGL3CSE-IR1$_{mutated}$); C: HepG2 cells were transfected with pSG5-FXR and pSG5-RXR expression vectors and with the construct containing four copies of the CSE-IR1 [pGL3 (CSE-IR1)$_{4x}$]. Forty-eight hours after transfection, cells were stimulated with 25 μmol/L of DCA, LCA, CA, CDCA and 6E-CDCA for 18 h. Luciferase activity is shown as the ratio of luciferase to β-galactosidase activities. $^{a}P < 0.05$ *versus* not treated cells; D: Dose-dependent induction of Luciferase activity by 6E-CDCA. $^{a}P < 0.05$ *versus* not treated cells; E: Mutagenesis of CSE-IR1 results in a loss of activation by FXR ligands. HepG2 cells were transfected with pSG5-FXR and pSG5-RXR expression vectors and with pGL3 or pGL3 (CSE-IR1)$_{4x}$ or pGL3CSE-IR1$_{mutated}$. Forty-eight hours after transfection, cells were stimulated with 10 μmol/L of 6E-CDCA for 18 h. Luciferase activity is show as the ratio of luciferase to β-galactosidase activities. $^{c}P < 0.05$ *versus* not stimulated pGL3 transfected cells. $^{e}P < 0.05$ *versus* not stimulated pGL3 (CSE-IR1)$_{4x}$ transfected cells; F: Guggulsterone abolished the transactivation of the CSE-IR1 element. HepG2 cells co-transfected with pSG5-FXR and pSG5-RXR expression vectors and with pGL3 (CSE-IR1)$_{4x}$ were stimulated with 50 μmol/L of guggulsterone alone or in combination with 10 μmol/L of 6E-CDCA. $^{a}P < 0.05$ *versus* not treated cells. $^{g}P < 0.05$ *versus* 6E-CDCA stimulated cells. Data represent the mean ± SD of three experiments.

using the FXR antagonist guggulsterone (Figure 2F). As expected, the stimulation of HepG2 cells co-transfected with pSG5-FXR, pSG5-RXR and pGL3 (CSE-IR1)$_{4x}$ with guggulsterone at 50 μmol/L for 18 h resulted in robust repression of luciferase activity with respect to non-stimulated cells (Figure 2F, columns 1 and 2; $n = 3$, $P < 0.05$ *vs* control cells). Treatment with 6E-CDCA resulted in about a four-fold increase of luciferase activity (Figure 2F, columns 1 and 3; $n = 3$, $P < 0.05$ *vs* not treated cells), while the transactivation was reduced in cells stimulated with both 6E-CDCA and guggulsterone with respect to cells stimulated only with 6E-CDCA (Figure 2F, columns 3 and 4; $n = 3$, $P < 0.05$ *vs* 6E-CDCA stimulated cells). These data establish that the IR1 motif in the proximal human CSE promoter is a functional FXR response element.

CSE-IR1 site binds FXR

To determine whether the IR1 element binds FXR, we performed an EMSA using the following biotin-labeled probes: CSE-IR1, CSE-IR1$_{mutated}$ and FXRE-IR1. CSE-IR1 biotin-labeled probe was incubated with nuclear extracts prepared from HepG2 cells left untreated or treated with 6E-CDCA 10 μmol/L for 18 h. As shown in Figure 3A, CSE-IR1 binding was detected in HEPG2 wild-type cells and exposure to 6E-CDCA enhanced this binding (Figure 3A, lanes 2 and 3). We confirmed the specificity of this interaction by adding 50-fold excess of unlabeled oligo or 1 μg anti FXR primary antibody or 1 μg anti RXR primary antibody (Figure 3A, lanes 4, 5 and 6). These approaches resulted in a reduction of DNA binding of the nuclear extract to CSE-IR1 probe. The specificity of the FXR interaction to CSE-IR1 was also confirmed using the mutated probe, CSE-IR1$_{mutated}$, and the positive control, FXRE-IR1. DNA binding and supershift was completely abrogated using the CSE-IR1$_{mutated}$ probe, while the FXRE-IR1 probe caused same supershift as the CSE-IR1 probe (Figure 3A, lanes 7 and 8). To study the DNA-protein complex interaction within the context of chromatin, ChIP was performed using serum-starved HepG2 cells exposed to 6E-CDCA 10 μmol/L. As show in Figure 3B and C, qualitative and

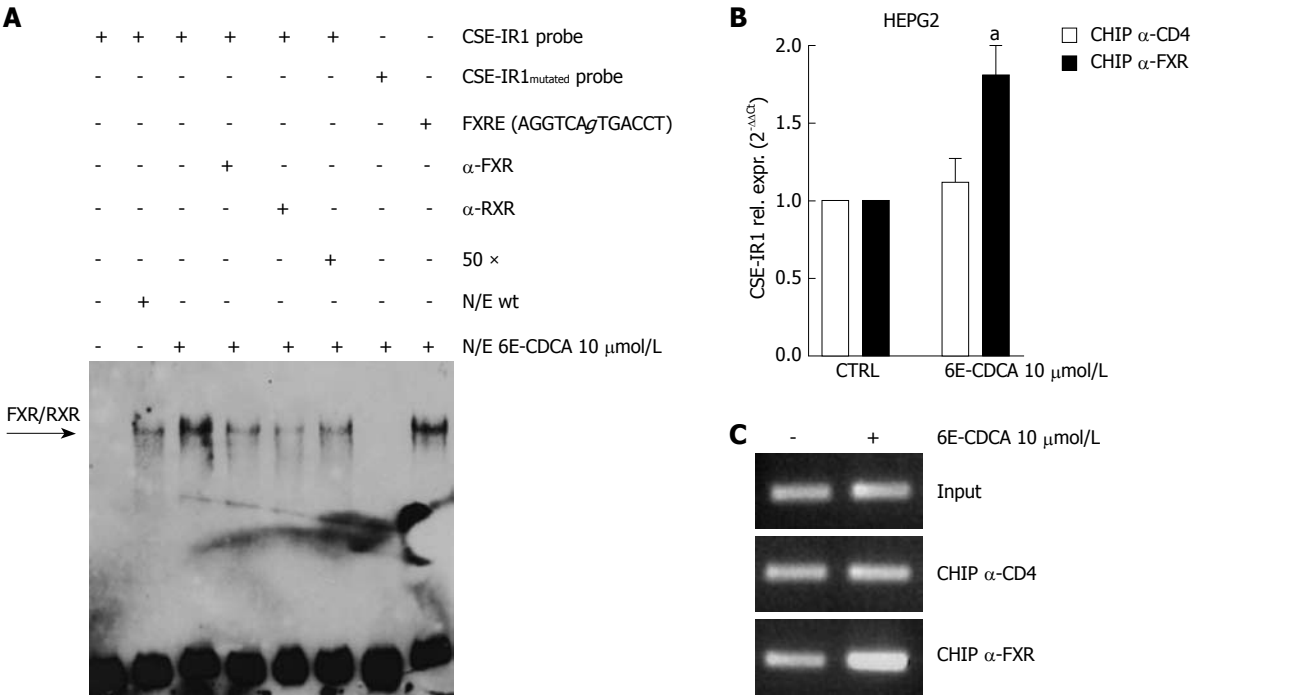

Figure 3 Activation of FXR regulates CSE expression. A: FXR/RXR bind to CSE-IR1 of the *CSE* gene. EMSAs were performed to analyze binding of FXR/RXR to the putative IR1 sequence in the CSE gene. CSE-IR1, CSE-IR1$_{mutated}$ and FXRE-IR1 probes, biotin-labeled, were used in this experiment. CSERE-IR1 probe was incubated with nuclear extracts from HepG2 cells not treated or treated with 6E-CDCA 10 μmol/L for 18 h. Competition experiments were performed using a 50-fold excess of unlabeled oligo or 1 μg of FXR antibody or 1 μg of RXR antibody. CSE-IR1$_{mutated}$ and FXRE-IR1 probes were incubated with nuclear extracts from HepG2 stimulated cells; B: CSE-IR1 site binds FXR in the context of intact chromatin structures. ChIP experiments were performed with HepG2 cells. Chromatin was prepared and immunoprecipitated with antibodies directed against FXR and CD4. CD4 antibody was used as a negative control. Real-time PCR of the immunoprecipitated DNA by using the primer pairs indicated in Table 1. Data represent the mean ± SD of three experiments. $^{a}P < 0.05$ *versus* not treated cells; C: Qualitative PCR of the immunoprecipitated DNA by using the primer pairs indicated in Table 1.

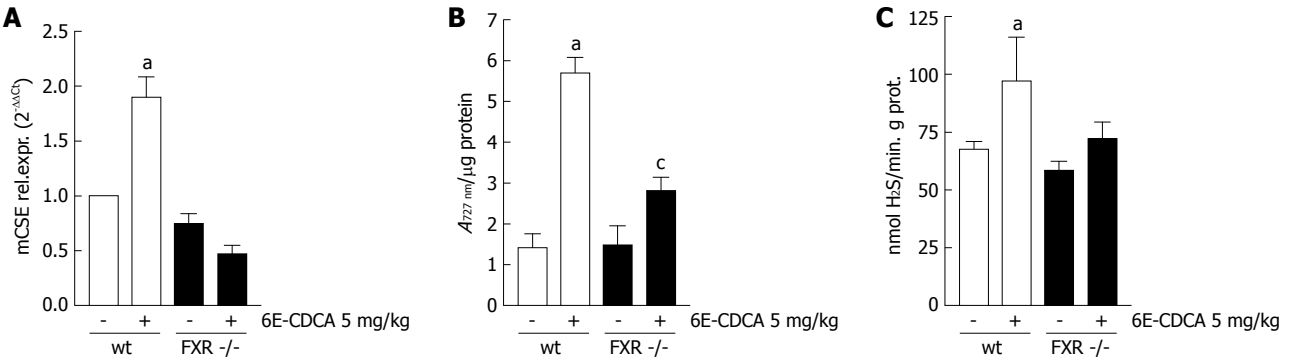

Figure 4 CSE expression/activity is regulated with an FXR ligand *in vivo*. A: FXR +/+ and FXR -/- mice were treated for 3 d with vehicle or with 6E-CDCA 5 mg/kg body weight. Total RNA from liver of FXR +/+ and FXR -/- mice was subjected to real-time PCR quantification of *CSE* gene expression. $^{a}P < 0.05$ *versus* FXR +/+ control mice; B: FXR +/+ and FXR -/- mice were treated for three days with vehicle or with 6E-CDCA 5 mg/kg body weight. Livers from FXR +/+ and FXR -/- mice were homogenized in cold PBS to evaluate CSE activity. $^{a}P < 0.05$ *versus* FXR +/+ control mice. $^{c}P < 0.05$ *versus* FXR -/- control mice; C: FXR +/+ and FXR -/- mice were treated for 3 d with vehicle or with 6E-CDCA 5 mg/kg body weight. Livers from FXR +/+ and FXR -/- mice were homogenized in cold PBS to evaluate H_2S production. $^{a}P < 0.05$ *versus* FXR +/+ control mice. Data represent the mean ± SD of six experiments.

quantitative PCR performed with primers flanking the CSE promoter containing the IR1 sequence, confirmed the binding of FXR at the *CSE* gene (Figure 3B; $n = 3$, $P < 0.05$ *vs* not treated cells). Thus, the functionality of this IR1 site was further confirmed in the context of intact chromatin structures.

CSE expression is induced by 6E-CDCA in vivo

To investigate whether FXR regulates *CSE* gene expression *in vivo*, wild-type and FXR -/- mice were administered with 6E-CDCA 10 mg/kg for 3 d and sacrificed to measure liver CSE expression, CSE activity and H_2S production. As show in Figure 4A, while an induction of CSE mRNA expression was seen in wild-type mice treated with 6E-CDCA ($n = 6$, $P < 0.05$ *vs* FXR +/+ control mice), this effect was not observed in FXR -/- mice, confirming that the *CSE* gene is a specific target of FXR. Interestingly, FXR activation by 6E-CDCA increased CSE activity in both wild-type and FXR -/- mice (Figure 4B; $n = 6$, $P < 0.05$ *vs* FXR +/+

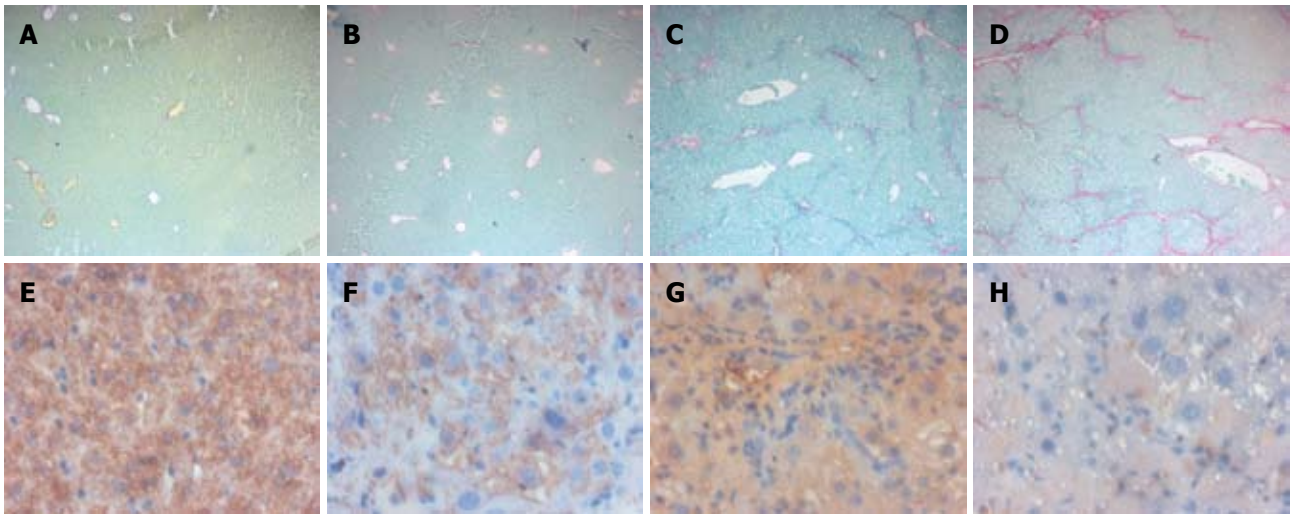

Figure 5 FXR loss of function sensitizes mice to CCl_4-induced liver fibrosis. A: Sirius red staining of liver section obtained from FXR +/+ mice; B: Sirius red staining of liver section obtained from FXR -/- mice; C: Sirius red staining of liver section obtained from FXR +/+ mice treated with CCl_4; D: Sirius red staining of liver section obtained from FXR -/- mice treated with CCl_4; E: Liver section stained with CSE monoclonal antibody obtained from FXR +/+ mice; F: Liver section stained with CSE monoclonal antibody obtained from FXR -/- mice; G: Liver section stained with CSE monoclonal antibody obtained from FXR +/+ mice treated with CCl_4; H: Liver section stained with CSE monoclonal antibody obtained from FXR -/- mice treated with CCl_4.

Table 2 Effect of loss of FXR on liver injury induced by 12 administrations of CCl_4 (6 wk)

	AST (U/L)	ALT (U/L)	Bilirubin (mg/dL)
FXR +/+ naive	127 ± 15	48 ± 8	0.015 ± 0.001
FXR +/+ CCl_4	250 ± 40[a]	369 ± 35[a]	0.118 ± 0.004[a]
FXR -/- naive	143 ± 10	79 ± 25[a]	0.068 ± 0.004[a]
FXR -/- CCl_4	740 ± 230[c,e]	354 ± 137[c]	0.252 ± 0.02[c,e]

Data are mean ± SD of six mice. [a]$P < 0.05$ *vs* FXR +/+ control mice; [c]$P < 0.05$ *vs* FXR -/- control mice; [e]$P < 0.05$ *vs* CCl_4 FXR +/+ mice.

control mice, $P < 0.05$ *vs* FXR -/- control mice). Taken together, these data suggest that while mRNA expression of CSE is regulated by an FXR-dependent mechanism, the induction of CSE activity by bile acids might be regulated by an FXR independent mechanism, possibly by TGR5 activation induced by bile acids. Finally, liver H_2S generation was significantly up-regulated by 6E-CDCA treatment in FXR +/+ mice but not in FXR -/- mice (Figure 4C; $n = 6$, $P < 0.05$ *vs* FXR +/+ control mice).

FXR loss of function sensitizes mice to CCl_4-induced liver fibrosis

We next investigated whether *in vivo* loss of FXR function sensitizes mice to development of liver fibrosis induced by administration of CCl_4. AST, ALT and bilirubin are commonly used biochemical markers of liver damage. As show in Table 2, the levels of ALT and bilirubin, but not of AST, in FXR -/- mice were much higher compared with the wild-type mice. *In vivo* administration of CCl_4 showed a significant increase of AST, ALT and bilirubin in FXR -/- mice with respect to FXR +/+ control mice (Table 2). Morphometric analysis of FXR +/+ and FXR -/- liver sections stained with Sirius red showed a normal distribution of collagen, with a variable amount in the portal tract and a thin rim around the terminal hepatic vein (Figure 5A and B), while histological evaluation of liver specimens obtained from FXR -/- mice treated with CCl_4 for 6 wk showed extensive perilobular fibrosis, resulting in an increase in the surface area of hepatic collagen in comparison with control FXR +/+ mice treated with CCl_4 (Figure 5C and D). Expression of CSE, observed by histochemical staining of liver sections, was reduced in FXR -/- mice compared with the wild-type mice (Figure 5E and F). Furthermore, FXR -/- mice administered with CCl_4 showed a significant reduction in CSE expression compared to FXR +/+ mice treated with CCl_4 (Figure 5G and H). Taken together, these data confirmed that mice lacking FXR are more likely to develop liver fibrosis, and that FXR loss of function correlates with reduction of CSE protein expression in the liver.

FXR activation restores H_2S production and CSE activity in a rodent model of liver cirrhosis

We then investigated whether *in vivo* administration of FXR ligands modulate CSE expression, the activity of the enzyme and H_2S production, in wild-type but not in FXR -/- mice administered with CCl_4. As show in Figure 6A, development of liver injury is associated with a significant reduction in CSE mRNA expression, in both the wild-type and FXR -/- mice treated with CCl_4 for 6 wk. In wild-type mice, administration of an FXR ligand resulted in a robust induction of CSE expression. This effect was not reproduced in FXR -/- mice, confirming the specificity of 6E-CDCA (Figure 6A; $n = 6$, $P < 0.05$ *vs* FXR +/+ control mice. $P < 0.05$ *vs* CCl_4 FXR +/+ mice). Similarly, we found that α1-collagen mRNA expression was down-regulated by 6E-CDCA in wild-type mice but not in FXR -/- mice (Figure 6B; $n = 6$, $P < 0.05$ *vs* FXR +/+ control mice. $P < 0.05$ *vs* CCl_4 FXR +/+ mice. $P < 0.05$ *vs* FXR -/- control mice). In addition, we found that liver CSE activity was down-regulated by CCl_4 administration in both FXR +/+ and FXR -/- mice, but this effect was reversed by treating the mice with 6E-CDCA (Figure 6C; $n = 6$, $P < 0.05$ *vs* FXR +/+ control mice. $P < 0.05$ *vs* CCl_4 FXR +/+ mice.

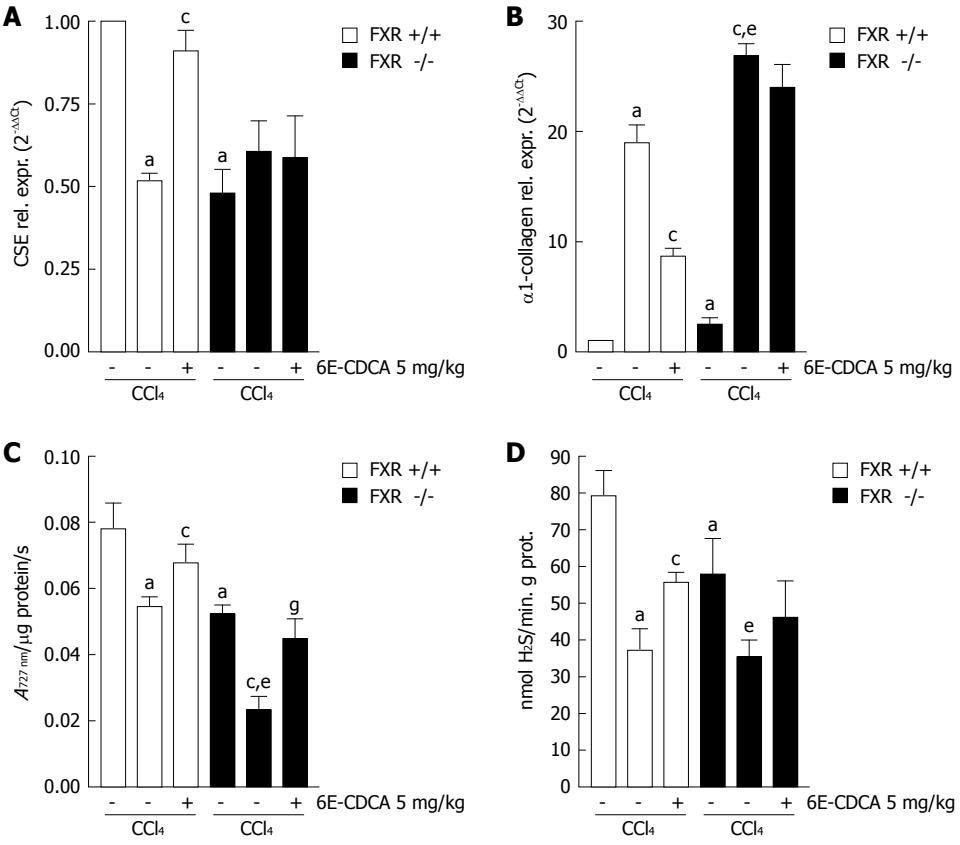

Figure 6 FXR activation induces *CSE* gene expression and regulated CSE activity in the liver with cirrhosis. FXR +/+ and FXR -/- mice were treated with CCl_4 and 6E-CDCA as described in the methods section. A and B: Quantitative real-time PCR of CSE mRNA and α1-collagen mRNA from FXR +/+ and FXR -/- liver homogenates; C: Liver CSE activity; D: Liver H_2S production. Data are mean ± SD of six mice. $^{a}P < 0.05$ *versus* FXR+/+ control mice. $^{c}P < 0.05$ *versus* CCl_4 FXR +/+ mice. $^{e}P < 0.05$ *versus* FXR -/- control mice. $^{g}P < 0.05$ *versus* CCl_4 FXR -/- mice.

$P < 0.05$ *vs* FXR -/- control mice. $P < 0.05$ *vs* CCl_4 FXR -/- mice). CCl_4 administration down-regulated liver H_2S production in both FXR +/+ and FXR -/- mice, while the administration of 6E-CDCA enhanced liver H_2S generation only in FXR +/+ mice (Figure 5D; $n = 6$, $P < 0.05$ *vs* FXR +/+ control mice. $P < 0.05$ *vs* CCl_4 FXR +/+ mice. $P < 0.05$ *vs* FXR -/- control mice).

FXR activation reduces portal perfusion pressure response to norepinephrine in cirrhotic rat liver

The reduction of CSE expression in the cirrhotic liver contributes to the development of increased intrahepatic resistance and portal hypertension. We therefore investigated whether *in vivo* administration of an FXR ligand modulates hepatic resistance in cirrhotic rats. As shown in Figure 7, the development of liver injury in rats reduced the expression of FXR and CSE (Figure 7A and B; $n = 6$, $P < 0.05$ *vs* control rats, $P < 0.05$ *vs* CCl_4 rats) while small heterodimer partner mRNA expression was unaffected (Figure 7C; $n = 6$, $P < 0.05$ *vs* CCl_4 rats). In contrast, CCl_4 administration up-regulated α1-collagen and αSMA mRNA (Figure 7D and E; $n = 6$, $P < 0.05$ *versus* control rats) Thus, treating CCl_4 rats with 6E-CDCA resulted in a robust induction of *FXR*, *SHP* and *CSE* genes (Figure 7A-C; $n = 6$, $P < 0.05$ *vs* CCl_4 rats), as well as suppression of α1-collagen gene expression (Figure 7D; $n = 6$, $P < 0.05$ *vs* CCl_4 rats). The CSE activity was strongly down-regulated by administration of CCl_4 in rats and the treatment with 6E-CDCA led to an increase of this enzyme activity (Figure 7F; $n = 6$, $P < 0.05$ *versus* control rats; $P < 0.05$ *vs* CCl_4 rats). Furthermore, as shown in Figure 8, the expression of CSE and αSMA was also investigated at the protein level by Western blotting analysis. We found that the CSE protein was strongly down-regulated during liver injury and that 6E-CDCA treatment resulted in a robust induction of this enzyme (Figure 8A). In contrast, CCl_4 treatment up-regulated the pro-fibrogenetic marker αSMA and administration of 6E-CDCA resulted in a suppression of this protein (Figure 8B). We then investigated whether FXR activation by a synthetic ligand lowers portal pressure in rodent models of liver injury. Under basal conditions, portal pressure was significantly higher in cirrhotic rats compared with the control rats (Figure 9A; $n = 6$, $P < 0.05$ *vs* control rats). In the cirrhotic rats, treatment with 6E-CDCA significantly decreased the portal pressure (Figure 9A; $n = 6$, $P < 0.05$ *vs* CCl_4 rats). Finally, data shown in Figure 9B demonstrated that in livers with cirrhosis, norepinephrine produced a dose-dependent increase in the portal perfusion pressure compared with control rats (Figure 9B; $n = 6$, $P < 0.05$ *vs* control rats). In contrast, treatment with 6E-CDCA reduced the hyper-responsiveness of livers with cirrhosis to norepinephrine (Figure 9B; $n = 6$, $P < 0.05$ *versus* CCl_4 rats).

DISCUSSION

Portal hypertension is associated with changes in intrahepatic, systemic, and portosystemic collateral circulation[28,29]. Alterations in vasoreactivity (vasodilatation and vasoconstriction) play a central role in the pathogenesis of this condition by contributing to increased intrahepatic resistance, hyperdynamic circulation and expansion of the collateral circulation[28,29]. The molecular basis of the vascular abnormalities that contribute to development of

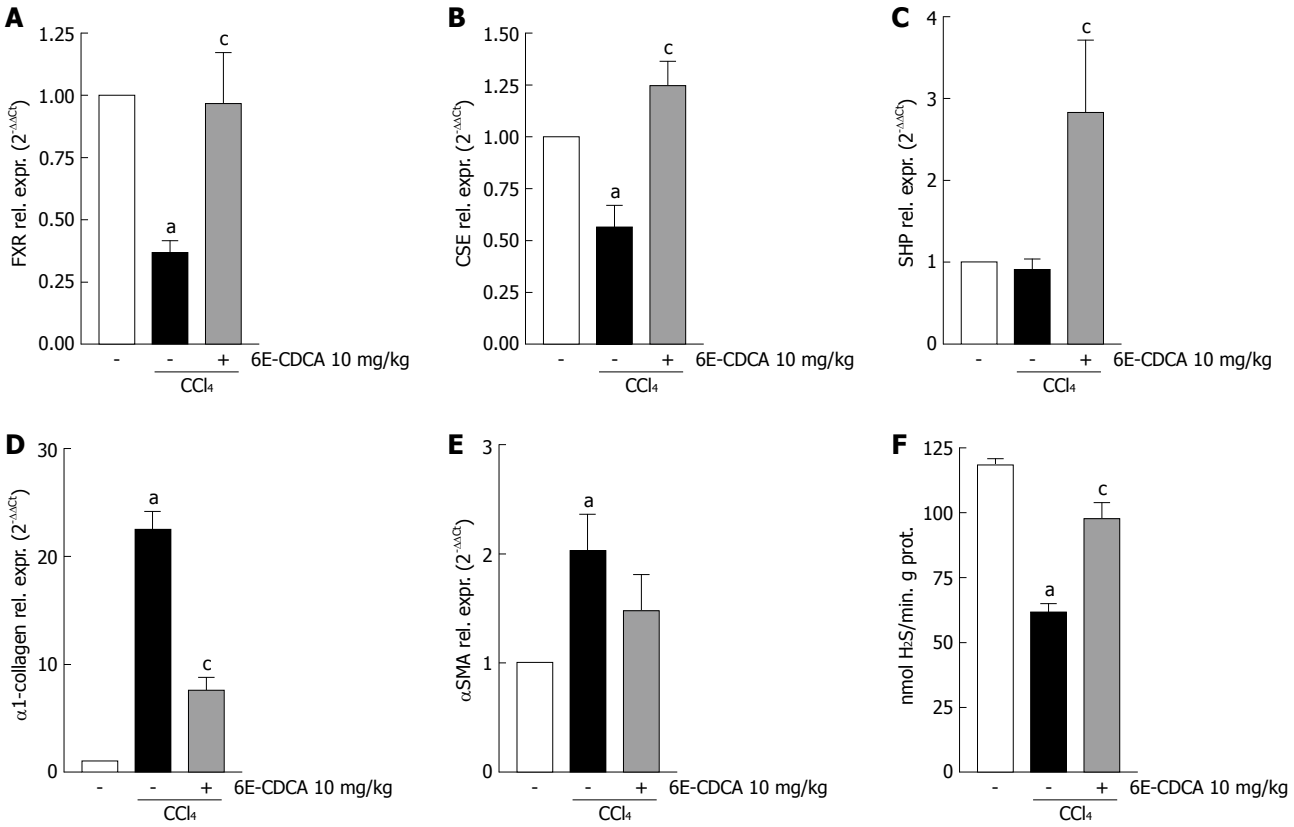

Figure 7 FXR activation induces both CSE mRNA expression and activity in rats with liver cirrhosis. Quantitative real-time PCR of (A) FXR mRNA, (B) CSE mRNA, (C) SHP mRNA, (D) α1-collagen mRNA, (E) αSMA mRNA from rats liver homogenates and (F) Rat liver CSE activity. Data are mean ± SD of six mice. $^aP < 0.05$ *versus* control rats. $^cP < 0.05$ *versus* CCl_4 rats.

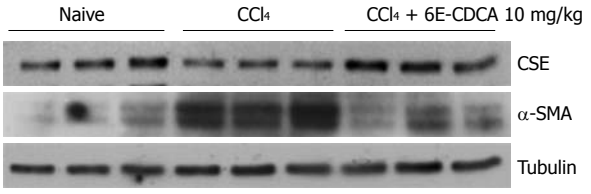

Figure 8 FXR activation induces CSE protein expression and reduces αSMA protein level in rat's liver with cirrhosis. Western blotting analysis of CSE, αSMA and tubulin on liver homogenates. From left to right: Lanes 1-3, liver samples from control rats; Lanes 4-6, liver samples from rats administered CCl_4; Lanes 7-9, liver samples from rats administered CCl_4 and 10 mg/kg 6E-CDCA.

portal hypertension are only partially identified[17,30-32]. A diminution in endothelial-nitric-oxide-synthase-derived NO production by liver sinusoidal cells contributes to this process by impairing the ability of hepatic microcirculation to vasodilate and therefore increases intrahepatic resistance[33]. We have previously described that along with NO, H_2S causes a direct relaxation of intrahepatic microcirculation, suggesting a physiological role for this gaseous mediator in regulating resistance of intrahepatic microcirculation. H_2S exerts a portal-pressure-lowering effect in normal rats as well as in rats rendered cirrhotic by CCl_4 administration, an experimental setting characterized by endothelial dysfunction of intrahepatic circulation and reduced generation of NO[9]. Finally, we have previously provided evidence that a robust reduction of H_2S generation takes place in cirrhotic rats and that this defect is linked to a decrease in the liver expression and activity of CSE, a key enzyme in the pathway that leads to generation of H_2S[17].

Little is known about the molecular mechanism responsible for the regulation of *CSE* gene expression and there is no evidence of the regulation of the *CSE* gene by nuclear receptors. FXR is one of the major nuclear receptors responsible for regulation of liver metabolism, therefore, we decided to study whether CSE expression in the liver was regulated by FXR. In the current study, we have shown, for the first time, that the liver expression of CSE is regulated by bile acids by means of an FXR mediated mechanism. By Western blotting, qualitative and quantitative PCR, as well as immunohistochemical analysis, we have shown that expression of CSE (mRNA and protein) in HepG2 cells is induced by treatment with bile acids and 6E-CDCA, a semi-synthetic FXR ligand. The molecular mechanism of the CSE activation by FXR was revealed by identifying a sequence in the 5' flanking region of the *CSE* gene, containing an element composed of two inverted repeats separated by one nucleotide (a potential IR1 binding site). Four copies of this IR1 binding site were cloned into the pGL3 vector containing the luciferase reporter gene, and in addition, a single copy of the IR1 binding site was mutated and cloned in the pGL3 vector. Co-transfection of HepG2 cells with FXR and RXR resulted in transactivation of the CSE promoter in the presence of an FXR ligand, while the mutation of the IR1 binding site and the treatment with an FXR antagonist, such as guggulsterone, abrogated this response. The FXR/RXR heterodimer bound specifically

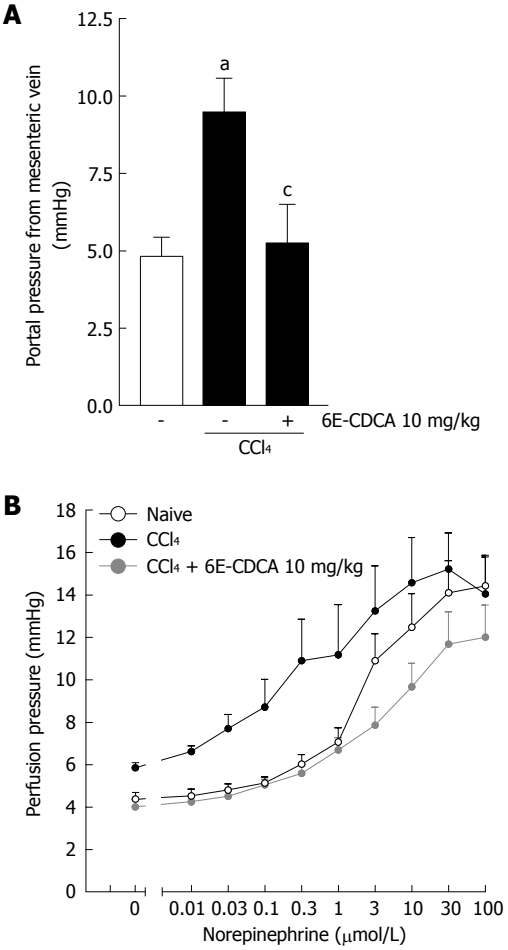

Figure 9 FXR activation reduces portal pressure in rat liver with cirrhosis. Data are mean ± SD of six mice. A: Basal portal pressure from mesenteric vein. $^{a}P < 0.05$ *versus* control rats. $^{c}P < 0.05$ *versus* CCl_4 rats; B: Effects of 6E-CDCA on pre-contracted rat liver with cirrhosis. $^{a}P < 0.05$ *versus* control rats. $^{c}P < 0.05$ *versus* CCl_4 rats.

to the CSE IR1 binding site, but not to the mutant form, as shown by a gel mobility shift assay using nuclear extracts from HepG2 cells not treated or treated with 6E-CDCA. The functionality of this IR1 site was also confirmed in the context of intact chromatin structures by a ChIP assay.

The role of FXR in the regulation of CSE has been further investigated *in vivo*, in mice harboring a targeted disruption of the *FXR* gene. These mice lack functional FXR and are unable to correctly regulate bile acids biosynthesis and excretion. Interestingly, when compared to the wild-type, FXR -/- mice displayed significantly lower levels of CSE and a reduced ability to produce H_2S. Similarly to the *in vitro* results, we found that in the normal liver, CSE expression was significantly increased when mice were fed a chow diet supplemented with 5 mg/kg body weight of 6E-CDCA, while the FXR ligand failed to up-regulate CSE mRNA expression in FXR knock-out mice. In contrast, administration of 6E-CDCA induced CSE activity in both wild-type and FXR knock-out mice. This finding suggested that the activity of the enzyme might be regulated by bile acids at the post-translational level, and a possible mechanism could be linked to the activation of the TGR5 induced phosphorylation cascade through the bile acids. We also confirmed that CSE liver expression was down-regulated in an animal model of liver damage induced by CCl_4 and that the reduction of H_2S generation seen in this model is likely to contribute to portal hypertension. One of the major findings of this study was the demonstration that mice lacking FXR are more likely to develop liver fibrosis and that loss of FXR function correlates with reduction of CSE protein expression in the liver. Treatment with an FXR ligand increased both CSE expression and activity in the cirrhotic liver, restoring the ability of injured livers to generate H_2S. These findings were not observed in cirrhotic FXR -/- mice treated with 6E-CDCA.

In addition to inhibition of NO formation by sinusoidal endothelial cells, homocysteine triggers an H_2S-sensitive contraction of hepatic stellate cells *in vitro*[18]. Contraction of presinusoidal myofibroblasts has relevance in regulating intrahepatic resistance and short-term administration of 6E-CDCA regulates CSE expression in normal mice, therefore, we investigated whether acute administration of an FXR ligand effectively modulates CSE expression in CCl_4 treated rats and whether this treatment was effective in correcting hepatic microcirculation hyper-responsiveness to norepinephrine. Despite the fact that even 3 d of administration of 6E-CDCA attenuated expression of 1-collagen and SMA mRNA, this anti-fibrotic activity did not completely explain the rapid re-induction of CSE expression in the liver that was associates with a restored ability to generate H_2S and a robust attenuation of hyper-responsiveness of cirrhotic livers to norepinephrine. The ability of the FXR ligand to lower portal pressure and to correct the enhanced vasomotor activity is consistent with the finding that perfusion of cirrhotic livers with H_2S attenuates the endothelial dysfunction that takes place in injured livers.

In conclusion, we have shown that CSE, a key enzyme in the trans-sulfuration pathway, is an FXR-regulated gene. Despite the fact that the level of expression/function of FXR in chronic liver disorders is still unknown, FXR is severely down-regulated in several models of liver injury. Reduction of FXR-regulated genes might contribute to the metabolic dysfunction that takes place in advanced cirrhosis. By linking the deficiency of CSE to the FXR activity the present study provides a new molecular explanation of the pathophysiology of portal hypertension. It also proposes the concept that FXR agonists might correct for the altered generation of endogenous hepatic vasodilators that takes place in chronic liver diseases.

COMMENTS

Background

Portal hypertension is primarily caused by the increase in resistance to portal outflow and an increase in splanchnic blood flow. Alterations in systemic and liver vasoreactivity play a central role in the pathogenesis of this condition by contributing to increased intrahepatic resistance, hyperdynamic circulation and expansion of the collateral circulation. Nitric oxide and hydrogen sulfide (H_2S) cause a direct relaxation of intrahepatic microcirculation suggesting a physiological role for these gaseous mediators in regulating resistance of intrahepatic microcirculation.

Research frontiers

Understanding of the pathophysiology of portal hypertension is essential in the development of new pharmacological treatment of this condition.

Innovations and breakthroughs

Farnesoid X receptor (FXR) is a bile acid sensor and upon activation it reduces the conversion of cholesterol into bile acids and increases bile acid excretion from hepatocytes by activating canalicular transporters. The authors demonstrate that cystathionase, a key enzyme for H_2S production, is an FXR regulated gene.

Applications

FXR agonists might correct for the altered generation of endogenous hepatic vasodilators that takes place in chronic liver diseases.

Peer review

The manuscript by Renga *et al* is a comprehensive study demonstrating the effect of FXR activation by bile acids on the expression of cystathione-γ-lyase and subsequent hydrogen disulfide production. Furthermore, mice lacking the FXR are more susceptible to the liver damage induced by CCl_4. This is a thorough and well-written manuscript that highlights important bile acid signaling events.

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Involvement of 90-kuD ribosomal S6 kinase in collagen type Ⅰ expression in rat hepatic fibrosis

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Abstract

AIM: To investigate the relationship between 90-kuD ribosomal S6 kinase (p90RSK) and collagen type Ⅰ expression during the development of hepatic fibrosis *in vivo* and *in vitro*.

METHODS: Rat hepatic fibrosis was induced by intraperitoneal injection of dimethylnitrosamine. The protein expression and cell location of p90RSK and their relationship with collagen type Ⅰ were determined by co-immunofluoresence and confocal microscopy. Subsequently, RNAi strategy was employed to silence p90RSK mRNA expression in HSC-T6, an activated hepatic stellate cell (HSC) line. The expression of collagen type Ⅰ in HSC-T6 cells was assessed by Western blotting and real-time polymerase chain reaction. Furthermore, HSCs were transfected with expression vectors or RNAi constructs of p90RSK to increase or decrease the p90RSK expression, then collagen type Ⅰ promoter activity in the transfected HSCs was examined by reporter assay. Lastly HSC-T6 cells transfected with p90RSK siRNA was treated with or without platelet-derived growth factor (PDGF)-BB at a final concentration of 20 μg/L and the cell growth was determined by MTS conversion.

RESULTS: In fibrotic liver tissues, p90RSK was over-expressed in activated HSCs and had a significant positive correlation with collagen type Ⅰ levels. In HSC-T6 cells transfected with RNAi targeted to p90RSK, the expression of collagen type Ⅰ was down-regulated (61.8% in mRNA, $P < 0.01$, 89.1% in protein, $P < 0.01$). However, collagen type Ⅰ promoter activity was not increased with over-expression of p90RSK and not decreased with low expression either, compared with controls in the same cell line ($P = 0.076$). Furthermore, p90RSK siRNA exerted the inhibition of HSC proliferation, and also abolished the effect of PDGF on the HSC proliferation.

CONCLUSION: p90RSK is over-expressed in activated HSCs and involved in regulating the abnormal expression of collagen type Ⅰ through initiating the proliferation of HSCs.

Key words: 90-kuD ribosomal S6 kinase; Collagen type Ⅰ; Hepatic fibrosis; Hepatic stellate cell; RNAi

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INTRODUCTION

Hepatic fibrogenesis is a response to injury in the liver. It is characterized by both a quantitative and qualitative change in the extracellular matrix (ECM), within which

collagen type I predominates[1,2]. The activated hepatic stellate cell (HSC) is primarily responsible for excessive collagen deposition during liver fibrosis[3-6]. Recently, multiple cellular signals, especially extracellular signal-regulated kinase 1 and 2 (ERK1/2), have been reported to be involved in the process of activation of HSCs by increasing protein phosphorylation and up-regulation of gene transcription[7-9]. 90-kuD ribosomal S6 kinase (p90RSK) is an important downstream substrate of ERK1/2. p90RSK itself interacts with numerous substrates in the cytoplasm and nucleus, and is involved in gene expression, protein synthesis, cell survival, cell cycle proliferation and progression[10-13]. p90RSK has been implicated in the pathogenesis of some tumors and some other chronic diseases[14]. However, the role of p90RSK in hepatic fibrosis has not yet to be fully elucidated. It is known that in rat hepatic fibrosis, p90RSK is significantly up-regulated in association with elevated collagen type I levels[15]. However, the relationship between p90RSK and collagen type I , including any regulatory effects of p90RSK on the expression of collagen type I , is elusive.

Hence the present study was undertaken to explore the relationship between p90RSK and collagen type I expression in the fibrotic liver.

MATERIALS AND METHODS

Animal model

Male adult Sprague-Dawley rats weighing 250 ± 12.3 g were purchased from the Centre of Experimental Animals in Jinling Hospital. The rats received intraperitoneal injections of dimethylnitrosamine (DMN) (Sigma, Saint-Quentin Fallavier, France) at 10 mg/kg body weight (n = 30) or 0.9% sodium chloride (n = 10) thrice a week as previously described[16]. The rats were injected for 1 wk (n = 10), 2 wk (n = 10), and 3 wk (n = 10), and were sacrificed 3 d after the last administration. At the time of sacrifice, a hepatectomy was performed and liver tissue samples were fixed in 10% buffered formalin and embedded in paraffin. The experimental protocol was approved by the Institutional Animal Care committee of Jinling Hospital.

Immunofluorescent staining

Liver sections were blocked with 5% normal goat serum after fixing and then simultaneously incubated with both monoclonal anti-p90RSK (1:200 dilution, BD Biosciences, San Jose, CA, USA) and polyclone anti-collagen type I (1:50 dilution, Rockland, Gilbertsville, PA, USA), or polyclonal antibody of α-smooth muscle actin (α-SMA) (1:100 dilution, Rockland, Gilbertsville, PA, USA) prepared in phosphate-buffered saline (PBS). The sections were incubated overnight at 4℃ or 1 h at room temperature and then washed with PBS. Sections were then simultaneously incubated with fluorescein isothiocyanate (FITC)-conjugated secondary antibody (1:100 dilution, Jackson Immunoresearch Laboratories, West Grove, PA, USA) and rhodamine-conjugated secondary antibody (1:200 dilution, Jackson Immunoresearch Laboratories) for 30 min at 37℃ in the dark. After extensive washing with PBS, the slides were mounted in a drop of Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA) to reduce photobleaching. Control experiments were performed in parallel with the omission of one of the primary antibodies. For double-staining experiments, both primary antibodies were produced in the different species.

Confocal microscopy and image analysis

Antibody labeling was examined under a Zeiss LSM-510 laser scanning confocal microscope. Optical slices (1.8 μm) were taken perpendicular to the liver section. A 488-nm argon laser was used in combination with a 499/505-530-nm excitation/emission filter set for fluorescence examination. For rhodamine, the 543-nm helium neon laser was used with a 543-nm excitation filter and 560-nm emission filter. Simultaneous images of FITC or rhodamine were captured from the same optical section. The captured images were then pseudocolored: red for rhodamine and green for FITC. Regions of co-localization appeared in yellow, reflecting the additive effect of superimposing green and red pixels. Image analysis was performed using the standard system operating software provided with the Zeiss LSM-510 series microscope.

Design of p90RSK siRNA and cell transfection

The RNAi targeting the p90RSK mRNA was designed by the software on the www.ambion.com. Forward oligo: TCGACAAAAGAGATCCCTCCGAAGTTCGCTTC GGAGGGATCTCTTTTTTTT. Reverse oligo: CTAG AAAAAAAAGTAGATCCCTCCGAAGCGAACTT CGGAGGGATCTCTTTTG. The vector of p90RSK siRNA was constructed using standard techniques[17]. p90RSK siRNA fragments and the vector pAVU6+27 were ligated, and the constructed plasmid with p90RSK siRNA was referred as pAVU6-siRSK. The activated cell line HSC-T6, a kind gift from Dr. Friedman (Mount Sinai School of Medicine), was cultured as previously described[18], and transfected with pAVU6-siRSK or empty plasmid pAVU6+27 by lipofectamine reagents (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions.

RNA extraction and real-time polymerase chain reaction (RT-PCR)

Total RNA was isolated from HSC-T6 cells transfected with pAVU6-siRSK or pAVU6+27 respectively, using Trizol in accordance with the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). One microgram of total RNA was then treated with DNAse for 30 min at 37℃. Reverse transcription was performed using the Omniscript RT kit (Qiagen, Valencia, CA, USA) and random primers (Promega, Madison, WI, USA). RT-PCR for rat p90RSK1 and collagen type I were performed using the ABI Prism 7700 Sequence Detection System, the Taqman universal PCR Master Mix, and assay-on-demand probes and primers (Shanghai Shengong Ltd.,

Table 1 RT- PCR primers

	Forward	Reverse
P90RSK	5′-TCTCTGTCCAGCG GCGGGTGAGGA-3′	5′-GCATTCACAGCG CCCATGCGCAG-3′
Collagen I	5′-CCAGCCGCAAAG AGTCTACATGTC-3′	5′-TCACCTTCTCAT CCCTCCTAA-3′
18S RNA	5′-GTCTGTGATGC CCTTAGATG-3′	5′-AGCTTATGACC CGCACTTAC-3′

Shanghai, China), according to standard protocols. The primers in RT-PCR are presented in Table 1. Parameters for baseline and threshold-cycle (C_t) settings were kept constant for each gene. To calculate ΔC_t, the C_t value for each target gene was standardized against that for the internal rRNA (18S) control probe.

Western blotting

Rat HSCs were lysed in 1 × sample buffer (62.5 mmol/L Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, 1 mmol/L Na_3VO_4). Ten micrograms of each sample were subjected to SDS-PAGE (7.5%-15%) and then transferred onto an Immobilon P membrane (Millipore Corp., Bedford, MA, USA). After blocking non-specific binding sites, the filters were incubated in Tween PBS at 4℃ for 16 h with one of the following antibodies: (1) mouse monoclonal antibodies directed against rat p90RSK (1:200) (BD); or (2) rabbit polyclonal antibody against rat collagen I (1:1000). Revelation was performed by a chemiluminescence-based method (ECL; Amersham Pharmacia Biotech, San Francisco, CA, USA).

Reporter assays

COL1A1 promoter (-378 to -340 bp)-luciferase reporter constructs were kindly provided by Dr. Huang (Nanjing Medical University). HSC-T6 cells (4×10^6), were electroporated (270 V, 950 μF) with 10 μg of the COL1A1-luciferase reporter and 2 μg of a Renilla luciferase expression construct (Promega), alone or in combination with pAVU6-siRSK, pAVU6+27 (empty vector), pMT2·RSK1 or pMT2 (empty vector) expression construct, respectively. HSC-T6 transfection efficiency was monitored by electroporation of a green fluorescent protein expression construct (10 mg). The relative luciferase value (RLV) was defined as the ratio of the luciferase activity divided by the activity of Renilla luciferase in transfected cell lysates. The RLV of unstimulated cultures was given the arbitrary value of 1. Each experiment was repeated a minimum of three times.

Analysis of HSC proliferation

Cell growth curves of HSC-T6 cells transfected with pAVU6-siRSK or control plasmid pAVU6+27 were analyzed by MTS conversion. Furthermore, to examine the effect of p90RSK siRNA on HSC proliferation induced by platelet-derived growth factor (PDGF), rhPDGF-BB (Boehringer Mannheim Co., Mannheim, Germany) was added to the medium at a final concentration of 20 μg/L in HSC-T6 cells transfected with pAVU6-siRSK or control plasmid pAVU6+27; cell growth was determined by MTS conversion as mentioned. The absolute number of HSCs in different groups by counting cells under microscopy after staining was also measured at the same time.

Statistics analysis

Statistical Package for the Social Sciences (version 10.0 for Windows; SPSS, Chicago, IL, USA) was used for statistical analysis. The calculation of Spearman's rank correlation coefficient was used to assess the relationship between quantitative parameters. Student's *t* test and the Mann-Whitney *U* test were used to compare data from different treatment groups. Data are expressed as mean ± SE. Differences were considered significant when *P* was less than 0.05.

RESULTS

Expression and relation of p90RSK with collagen type I in DMN-treated rats

Immunofluorescent double-staining showed abundant expression of collagen type I and p90RSK in the fibrotic liver (Figure 1A and B). However, in normal liver, only a little collagen type I could be observed and no p90RSK was detected (Figure 1D and E). Image analysis showed that both of p90RSK and collagen type I were up-regulated simultaneously, but these two signals did not co-localize (Figure 1C and F).

Cellular localization of p90RSK in DMN-treated rats

α-SMA, a typical marker of activated HSCs, was selected for this study to determine the cellular localization of p90RSK in fibrotic liver. The localization of p90RSK and α-SMA was visualized by immunofluorescent double labeling and laser scanning confocal microscopy. Image analysis showed a diffused distribution of p90RSK throughout the fibrotic liver (Figure 2A), and a similar distribution was observed with α-SMA staining (Figure 2B). When the two images merged, p90RSK showed a very high degree of co-localization with α-SMA throughout the fibrotic liver (Figure 2C).

Identification of p90RSK siRNA

The recombinant pAV-siRSK was identified by enzyme digestion (Figure 3) and sequencing, which showed that digestion product of pAV-siRSK was about 350 bp, compared with 300 bp production of pAVU6+27. The sequencing result showed siRSK was 52 bp.

Regulation of p90RSK siRNA on collagen type I

The RT-PCR experimental conditions were optimized to obtain an efficacy up to 90% of standard curves. When p90RSK mRNA in HSC-T6 cells was silenced using RNAi and the mRNA of p90RSK and collagen type I examined in HSC-T6 cells transfected with pAVU6-siRSK or empty pAVU6+27, there was an obvious reduction of 72.6% in p90RSK mRNA levels within HSC-T6 cells transfected with pAVU6-siRSK.

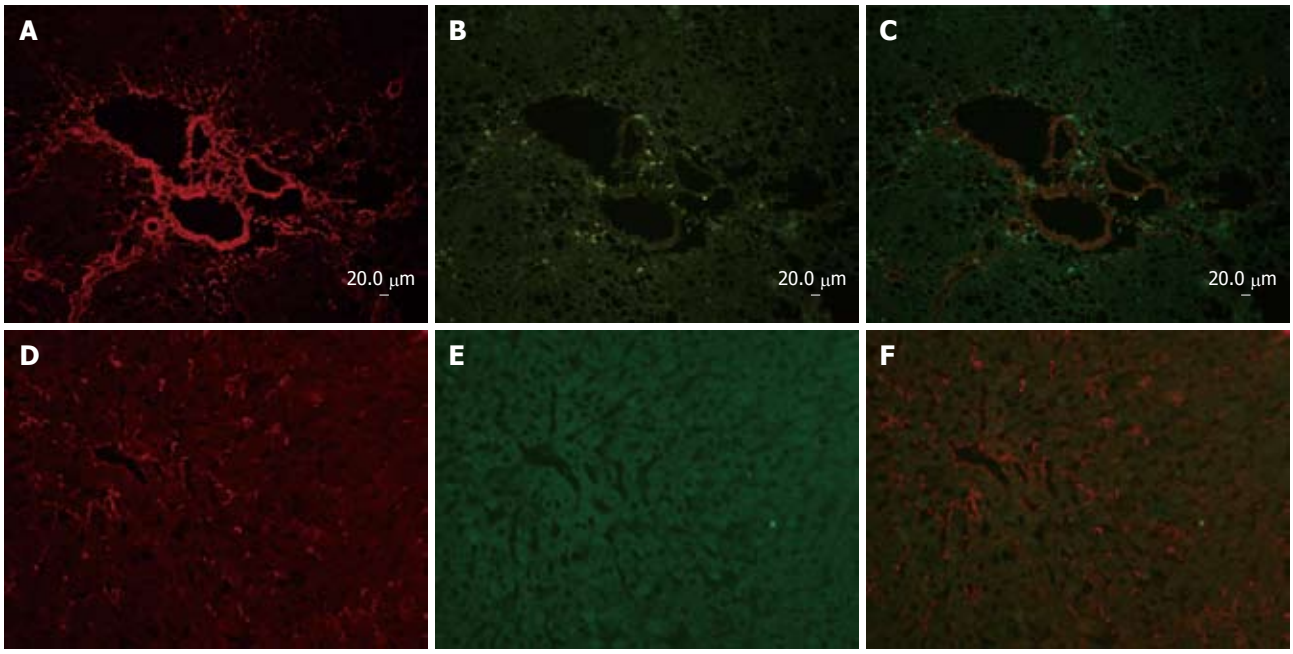

Figure 1 Co-immunofluoresence of p90RSK and collagen type Ⅰ in fibrotic liver and normal liver. Sections (A-C) are from rat liver with intraperitoneal injection of DMN for 3 wk, and sections (D-F) are from normal livers as control. Sections of fibrotic liver mostly demonstrate that collagen type Ⅰ (rhodamine) and p90RSK (FITC) immunoreactivity were both present around central veins as well as in the interstitium, and up-regulated in fibrotic liver. Sections of normal liver mostly demonstrate that collagen type Ⅰ (rhodamine) immunoreactivity was less in normal liver than in fibrotic liver and p90RSK (FITC) was hardly observed in nomal liver simultaneously.

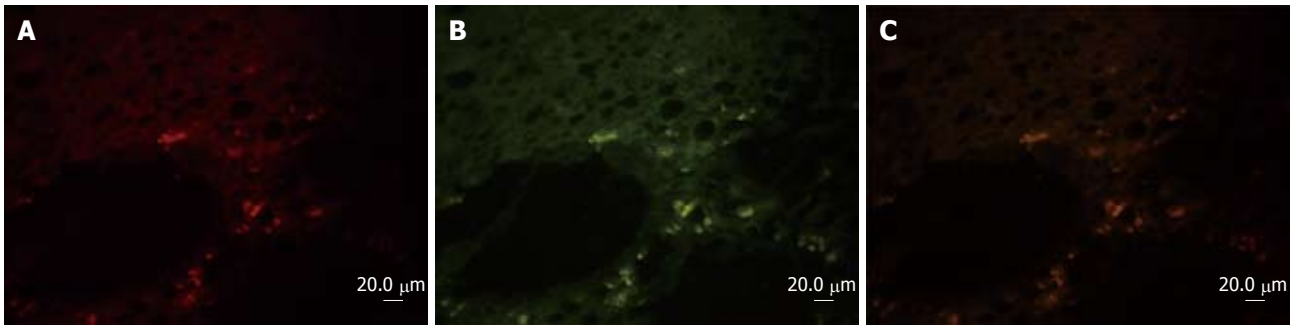

Figure 2 Cellular localization of p90RSK in fibrotic liver and co-immunofluoresence and confocal microscopy of p90RSK and αSMA in fibrotic liver. A: α-SMA (rhodamine)-positive cell represent the activated HSC, which deposited in interstitium; B: P90RSK (FITC)-positive cell were also present in interstitium; C: The yellow areas on the merged image show co-localization of α-SMA and p90RSK.

There was also a reduction of 61.8% in collagen type Ⅰ ($P < 0.01$, Figure 4).

The protein level of p90RSK and collagen type Ⅰ were examined by Western blotting in HSC-T6 cells transfected with pAVU6-siRSK or empty pAVU6+27. The protein level of p90RSK and collagen type Ⅰ was reduced to 75.6% and 89.1%, respectively, after RNAi ($P < 0.01$, Figure 5).

Effect of p90RSK siRNA on collagen type Ⅰ promoter activity

Collagen type Ⅰ is a heterotrimer composed of two coordinately expressed α1 chains and one α2 chain. They are encoded by distinct genes, COL1A1 and COL1A2, respectively[19]. The -378 to -340 region of the COL1A1 promoter exploited in this study is the site of convergence of different stimuli to modulate the gene transcription[20]. In this study, we observed that over-expression of p90RSK had little effect on activity of this region. Similarly, silencing of p90RSK expression did not decrease its activity either (Figure 6). The results showed that p90RSK did not work on COL1A1 promoter. Herein, we identified p90RSK could not alter transcriptional activity of collagen type Ⅰ in HSCs.

Effect of p90RSK siRNA on HSC proliferation

PDGF is a well known ligand able to elicit proliferation as well as to operate through ERK1/2 pathway and the most potent mitogen for HSCs *in vitro*. To further investigate the role of the p90RSK in the mitogenic effects on HSCs, we used the RNAi strategy, to produce the post-transcriptional gene expression silencing of p90RSK in HSCs. In accordance with previous studies, our data showed that p90RSK siRNA significantly inhibited the proliferation of HSC-T6 (Figure 7A) and also abolished the effect of PDGF-BB on HSC proliferation (Figure 7B).

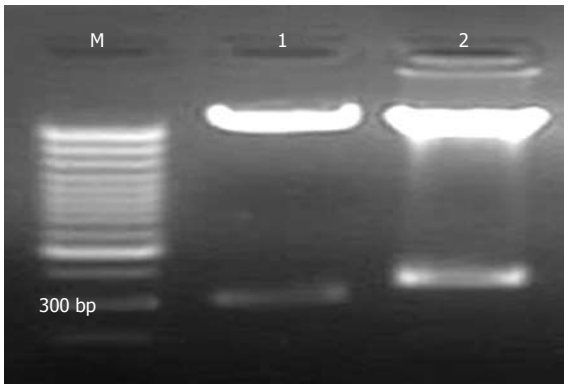

Figure 3 Agarose gel electrophoresis of restriction enzyme digestion of pAV-siRSK. M: 1 kb marker; 1: Restriction enzyme digestion product of pAVU6+27 was about 300 bp; 2: Restriction enzyme digestion product of pAV-siRSK. The siRNA of p90RSK was designed of 52 bp.

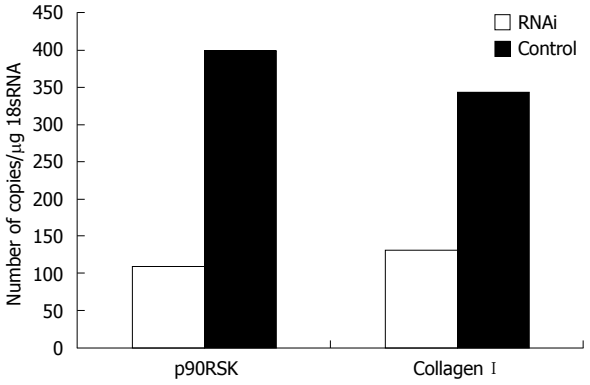

Figure 4 RT-PCR assessment of p90RSK and collagen type I in HSC-T6 transfected with or without pAVU6-siRSK. Quantification of p90RSK and collagen type I normalized to 18S RNA in HSC-T6 cells transfected with pAVU6-siRSK decreased 72.6% and 61.8%, respectively, compared with control ($P < 0.01$). Results are expressed as mean ± SE of three separate experiments.

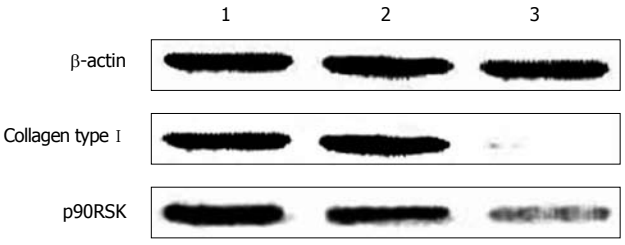

Figure 5 Western blotting analysis of p90RSK, collagen type I in HSC-T6 cells transfected with or without pAVU6-siRSK. β-actin provided as an inner control. 1: Normal HSC-T6 cells; 2: HSC-T6 transfected with empty plasmid; 3: HSC-T6 transfected with pAVU6-siRSK. With p90RSK siRNA, the expression of p90RSK decreased 75.6% compared with controls, and the expression of collagen type I decreased 89.1% accordingly ($P < 0.01$). Results are expressed as mean ± SE of three separate experiments.

DISCUSSION

Northern blot and immunohistochemical analysis has previously demonstrated that the expression of p90RSK has a significant correlation with that of collagen type I during the development of hepatic fibrosis[15]. In that study, the measurements were performed separately, preventing the determination of any spatial relationship between p90RSK and collagen

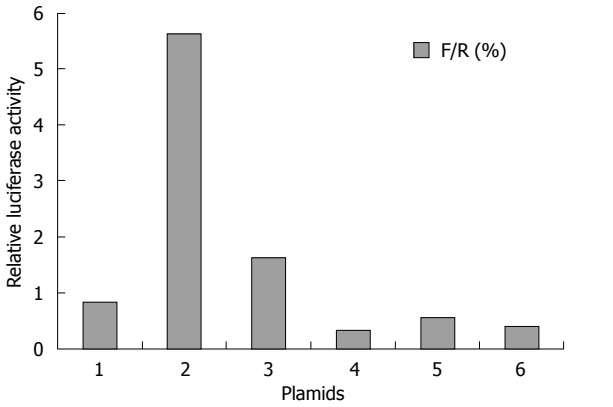

Figure 6 Effect of p90RSK on collagen type I promoter activity. HSCs transfected with the pAVU6-siRSK (bar 3, to decrease p90RSK expression), pMT2·RSK1 (bar 5, to increase p90RSK expression) showed no alteration of collagen type I promoter activity compared to control cells sham-transfected with pAVU6+27 (bar 4), pMT2 (bar 6), respectively. HSCs transfected with collagen type I luciferase reporter construct (bar 1) as normal control and Renilla luciferase expression construct (bar 2) as positive control. After 24 h incubation, the luciferase activity was determined. Data represent luciferase activity relative to the control (bar 1) and are expressed as mean ± SD of three separate experiments.

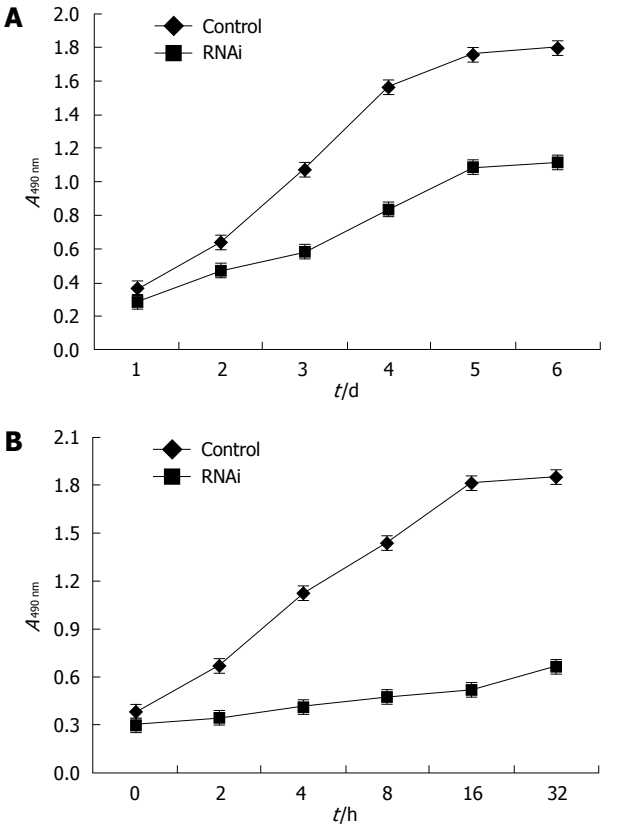

Figure 7 Effect of p90RSK siRNA on the proliferation of HSCs. A: p90RSK siRNA inhibited the proliferation of HSC-T6 cells. Cell growth curves of the recombinant cells with or without p90RSK siRNA were analyzed by MTS conversion; B: p90RSK siRNA abolished the effect of PDGF-BB on the proliferation of HSCs. rhPDGF-BB was added to the medium at a final concentration of 20 μg/L in HSC-T6 cells transfected with pAVU6-siRSK (RNAi), which showed decreased proliferative activity compared to sham transfected control cells (control). Each sample was tested in triplicate and error bars were included.

type I . Therefore, to ascertain whether there was any association between them, the expression of p90RSK and collagen type I was measured simultaneously by immunofluorescent double-staining and confocal microscopy. The results indicate that both p90RSK and collagen type I increase simultaneously in the same section of the fibrotic liver.

The activated HSC is the primary cell type in the liver responsible for the excess synthesis and deposition of ECM, within which collagen type I predominates[5,9]. It resides in the perisinusoidal space of Disse in the liver[21]. In our previous studies, p90RSK was observed in interstitial cells, which include activated HSCs and some other interstitial cells. To determine whether the over-expression of p90RSK was located in activated HSCs, α-SMA was employed as an HSC activation marker[21-23]. The result of confocal microscopy showed that p90RSK and α-SMA are co-localized within the interstitium. Hence, up-regulated p90RSK is located within activated HSC.

HSC-T6 is the immortalized rat HSC line, which retains all features of activated stellate cells, including expression of desmin, α-SMA, and glial acidic fibrillary protein, as well as collagen[24]. Because primary stellate cell cultures and isolation is extremely time-consuming, yields are modest, and there is considerable preparation-to-preparation variability, we used HSC-T6 cells to study the role of p90RSK *in vitro*. We observed that down-regulation of the post-transcriptional gene expression of p90RSK in the HSC-T6 cells, was achieved through the administration of p90RSK siRNA. Subsequently, the expression of collagen type I mRNA was significantly reduced, leading to a reduction of collagen type I in cell culture supernatant. This is in agreement with previous reports[15], and strengthens the evidence for p90RSK production having an influence on collagen type I expression in activated HSCs.

It is known that HSCs are directly involved in mediating the fibrogenic response in hepatic fibrosis. They become fibrogenic by synthesizing ECM proteins and activated HSCs proliferate, thereby amplifying the fibrogenic response[25]. It is becoming clear that both proliferative (i.e. PDGF)[17] and fibrogenic (i.e. transforming growth factor-β)[26] cytokines activate ERK1/2 signaling cascades in the development of hepatic fibrosis. p90RSK could be activated by the above stimuli and has widely distributed substrates[17,27-29]. The diversity of these stimuli and substrates suggests that p90RSK may be involved in the regulation of a wide range of cellular functions[11-13]. ERK1/2 has an important role in the signaling pathway that leads to the proliferation of HSCs. From this, it might be speculated that p90RSK, as a potent downstream substrate of the ERK1/2 signaling pathway, is involved in the fibrogenic activation of HSCs, or proliferation of HSCs, or both. Reporter assays designed to address the ability of p90RSK to regulate the activity of the collagen type I promoter were used to explore the role of p90RSK in the transcriptional induction of collagen type I gene expression in HSCs. The results showed that neither an increase nor decrease of p90RSK has any effect on the collagen type I promoter activity. Otherwise, the analysis of HSC proliferation showed that p90RSK siRNA significantly inhibited the proliferation of activated HSCs and also abolished the effect of PDGF-BB on that of HSCs. This suggests that p90RSK has no effect on the fibrogenic activation of HSCs, rather that p90RSK increases the collagen type I expression *via* the initiation of HSC proliferation. This observation is in line with the recent report that p90RSK phosphorylates C/EBPβ to inhibit activated HSC apoptosis in liver fibrosis[30].

Therefore, we conclude that p90RSK is over-expressed in activated HSCs and involved in the regulation of collagen type I expression through the initiation of HSC proliferation.

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We gratefully acknowledge Dr. Scott L Friedman (Professor in Mount Sinai School of Medicine) for kindly providing the cell line HSC-T6. We are grateful to Dr. Joseph Avruch (Professor of the Harvard Medical School) for the plasmid pMT2·RSK1 and Dr. David R Engelke (Professor of biological chemistry in University of Michigan) for the plasmid pAVU6+27.

COMMENTS

Background

Hepatic fibrogenesis is a response to injury in the liver. It is characterized by both a quantitative and qualitative change in the extracellular matrix, within which collagen type I predominates. The activated hepatic stellate cell (HSC) is primarily responsible for excessive collagen deposition during liver fibrosis. Recently, multiple cellular signals, especially extracellular signal-regulated kinase 1 and 2 (ERK1/2), have been reported to be involved in the process of activation of HSCs by increasing protein phosphorylation and up-regulation of gene transcription. However, the molecular mechanism is not fully elucidated.

Research frontiers

90-ku ribosomal S6 kinase (p90RSK) is an important downstream substrate of ERK1/2. p90RSK itself interacts with numerous substrates in the cytoplasm and nucleus, and is involved in gene expression, protein synthesis, cell survival, cell cycle proliferation and progression. p90RSK has been implicated in the pathogenesis of some tumors and some other chronic diseases. The authors' previous research has demonstrated that p90RSK is significantly up-regulated in rat hepatic fibrosis. However, the role of p90RSK in hepatic fibrosis has yet to be fully elucidated. In this study, the authors demonstrate that the overexpression of p90RSK could be a potential mechanism for mediating collagen type I expression.

Innovations and breakthroughs

Recent reports have highlighted the importance of p90RSK in cell proliferation and differentiation. In particular, p90RSK is required for cytostatic factor arrest in *Xenopus laevis* eggs. This is the first study to investigate the regulatory mechanism of p90RSK on collagen type I expression in rat HSCs.

Applications

This study may represent a future strategy for therapeutic intervention in the treatment of hepatic fibrosis.

Terminology

p90RSK is a serine/threonine kinase, which is the key substrate of the ERK1/2 signal pathway and involved in the phosphorylation of transcription factors, including nuclear factor-κB, c-Fos, Nur77, and cAMP response element-binding protein.

Peer review

The study by Yang *et al* investigated the relationship between p90RSK and

collagen type Ⅰ expression during the development of experimental hepatic fibrosis induced by dimethylnitrosamine. By also employing a number of experimental procedures and the T6 rat-model of immortalized HSCs, the authors conclude that p90RSK is over-expressed in activated HSCs and involved in the abnormal expression of collagen type Ⅰ, although collagen type Ⅰ promoter activity is not affected by either p90RSK over-expression or silencing. The study, of appreciable technical quality, is of potential interest for a reader interested in liver fibrosis. Data are mostly straightforward.

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ORIGINAL ARTICLES

Components of the mitogen-activated protein kinase cascade are activated in hepatic cells by *Echinococcus multilocularis* metacestode

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Author contributions: Lin RY originated the study, he performed most of the experimental work, analyzed the data and prepared the figures and the draft versions of the manuscript; Wang JH and Lu XM were involved in the collection, preservation and pathological identification of the human liver samples in Urumqi, China; Zhou XT contributed to the immunostainings and measurements performed on these samples; Mantion G and Wen H, hepatic surgeons, contributed to the design of the study, to the diagnosis, surgical treatment and follow-up of the patients with alveolar echinococcosis and supervised *in vivo* studies; Vuitton DA contributed to the design of the study, and interpretation of the data; Richert L was much involved in the interpretation of the data and revised all draft versions and the definitive version of the manuscript.
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Abstract

AIM: To explore the effect of *Echinococcus multilocularis* (*E. multilocularis*) on the activation of mitogen-activated protein kinase (MAPK) signaling pathways and on liver cell proliferation.

METHODS: Changes in the phosphorylation of MAPKs and proliferating cell nuclear antigen (PCNA) expression were measured in the liver of patients with alveolar echinococcosis (AE). MAPKs, MEK1/2 [MAPK/extracellular signal-regulated protein kinase (ERK) kinase] and ribosomal S6 kinase (RSK) phosphorylation were detected in primary cultures of rat hepatocytes in contact *in vitro* with (1) *E. multilocularis* vesicle fluid (EmF), (2) *E. multilocularis*-conditioned medium (EmCM).

RESULTS: In the liver of AE patients, ERK 1/2 and p38 MAPK were activated and PCNA expression was increased, especially in the vicinity of the metacestode. Upon exposure to EmF, p38, c-Jun N-terminal kinase (JNK) and ERK1/2 were also activated in hepatocytes *in vitro*, as well as MEK1/2 and RSK, in the absence of any toxic effect. Upon exposure to EmCM, only JNK was up-regulated.

CONCLUSION: Previous studies have demonstrated an influence of the host on the MAPK cascade in *E. multilocularis*. Our data suggest that the reverse, i.e. parasite-derived signals efficiently acting on MAPK signaling pathways in host liver cells, is actually operating.

Key words: *Echinococcus multilocularis*; Hepatic alveolar echinococcosis; Mitogen-activated protein kinase; Host-parasite interactions; Liver

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INTRODUCTION

Changes in the metabolic pathways involved in homeostasis and growth regulation of hepatic cells, and especially in the mitogen-activated protein kinase (MAPK) system, have been extensively studied in infectious/inflammatory conditions; of those, viral infections, and especially HBV and HCV in relation to hepatic carcinogenesis, have received most attention[1-3]. Very little is known about the capacity of helminth parasites and/or their components/secretions to influence liver cell homeostasis metabolic pathways. Actually, a few helminth parasites do affect the liver[4]. Among them, infection with *Echinococcus multilocularis* (*E. multilocularis*) larva (metacestode) affects primarily the liver and causes alveolar echinococcosis (AE) in intermediate hosts. It is an aggressive chronic parasitic infection that is characterized by a multivesicular structure surrounded by an extensive fibro-inflammatory host reaction[5]. In humans, who behave as accidental intermediate hosts, the severity of this life-threatening disease results from both a continuous asexual proliferation of the metacestode and an intense granulomatous infiltration around the parasite; the lesions behave like a slow-growing liver cancer. Invasion of biliary and vascular walls is another hallmark of this severe disease[6,7]. The ensuing fibrosis protects the patients against parasitic growth, but at the same time distorts the liver parenchyma[8-13]. Hepatomegaly is a usual symptom of AE; it has been ascribed to the liver regeneration which accompanies the pseudo-tumoral process[7]. However, unlike other forms of liver injury, e.g. from neoplasms, viral hepatitis or physical injury in which cell cycle regulatory genes have been extensively investigated[14,15], the cellular and molecular consequences of *E. multilocularis* infection on liver cells have never been studied.

It has been shown that the larval development of *E. multilocularis* is triggered by cell signaling originating from the intermediate host[16,17]. The phosphorylation of EmMPK1, a parasitic orthologue of the extracellular signal-regulated kinase (ERK) MAPK, is specifically induced in *in-vitro*-cultured *E. multilocularis* metacestode vesicles, in response to exogenous host serum, hepatic cells and/or human epidermal growth factor (EGF). The *E. multilocularis* metacestode is thus able to "sense" host factors which results in an activation of the parasite MAPK cascade[18]. The fact that tissue-dwelling *E. multilocularis* expresses signaling systems with significant homologies to those of the host raises the interesting question whether cross-communication between cytokines and corresponding receptors of host and parasite can occur during an infection, i.e. whether the parasite may also influence signaling mechanisms of host cells through the secretion of various molecules that might bind to host cell surface receptors. Such interactions could contribute to immunomodulatory activities of *E. multilocularis* or be involved in mechanisms of organotropism and/or in host tissue destruction or regeneration during parasitic development. Only gross changes in carbohydrate metabolism[19] and in protein/albumin secretion by liver cells[20] have been studied in experimental and *in vitro* models of *E. multilocularis* growth. To the best of our knowledge, no study has reported on the activation pattern of liver cell MAPK during *E. multilocularis* host infection. MAPKs are key regulators of cellular signaling systems that mediate responses to a wide variety of extracellular stimuli. MAPK signaling pathways, including c-Jun N-terminal kinase (JNK), p38 MAPK and ERK, play important roles in signal transduction from the cell membrane to the nuclear transcriptional factors; they cross-communicate and regulate the balance between cell survival and cell death in acute and chronic liver injury[21,22]. Generally, the JNK and p38 MAPK families appear to be pro-apoptotic, while the ERK pathway appears to be anti-apoptotic in mediating specifically cell growth and survival signals in many cell types[23]. The dynamic balance of their activities appears critical in acute liver injury such as viral hepatitis, drug- or toxin-induced toxicity or acute rejection after liver transplantation as well as in chronic liver injury[1,24]. For all these reasons we chose them as a first target.

The aim of the present study was thus to explore the influence of *E. multilocularis* metacestode on the activation of MAPK signaling pathways (ERK1/2, JNK and p38) and on liver cell proliferation. To reach this goal, we first studied the changes induced in the liver of patients with chronic AE, and then, the changes in hepatic cell cultures in contact *in vitro* with (1) *E. multilocularis* vesicle fluid (EmF), and (2) *E. multilocularis*-conditioned medium (EmCM).

MATERIALS AND METHODS

Tissue samples

The diagnosis of AE was established on positive serology with ELISA using crude *E. multilocularis* and Em2 antigens[25] and characteristic liver lesions observed at ultrasound and CT-scanning, and confirmed by histological examination of the lesions. To demonstrate the influence of *E. multilocularis* lesions on the surrounding hepatic cells, paired liver specimens (volume: 0.5 cm^3 each) were obtained at surgery by an experienced surgeon from AE patients at the Liver Surgery and Transplantation Units of the University Hospital, Besancon, France (one patient), and of 1st Teaching Hospital, Xinjiang Medical University (TH-XMU), Urumqi, China (four patients). In each patient, one specimen was taken close to the parasitic lesions (i.e. 0.5 cm from the macroscopic changes due to the metacestode/granuloma lesion, thus avoiding liver contamination with infiltrating immune cells and parasitic tissue), and one was taken distant from the lesions (i.e. in the non-diseased lobe of the liver whenever possible, or at least at 10 cm from the lesion), according to a previously described procedure[11]. Absence of contamination by the parasitic lesions was checked on all samples by histological examination. The patients gave their informed consent for the use of tissue samples for research, as part of a research project approved by the "*Comité*

Régional de Protection des Personnes en Recherche Biomédicale" de Franche-Comté, according to the French regulation, and by the Ethical Committee of TH-XMU. The liver samples were homogenized in ice-cold lysis buffer as previously described[26] and homogenates were clarified by centrifugation at 10000 *g* for 10 min at 4℃. Protein concentration was estimated by the BCA Assay kit (Sigma, Steinheim, Germany). Samples were stored at -80℃ until use.

EmCM and EmF

The EmCM without serum was kindly provided by Klaus Brehm (Institute of Hygiene and Microbiology, University of Würzburg, Germany) and was prepared as described previously[27] and stored at -80℃ until used.

EmF was extracted from vesicles in *E. multilocularis*-infected *Cricetulus migratorius* maintained at the Experimental Animal Research Laboratory of TH-XMU, according to the international guidelines for the maintenance of experimental animals for medical research. All procedures were carried out in a class Ⅱ laminar flow cabinet with appropriate protective clothing. The parasite material was removed from the peritoneal cavity under aseptic conditions, and was washed three times in phosphate buffered saline. The membrane was punctured with a 21-gauge needle connected to a 50-mL syringe. Fluid was withdrawn carefully until *E. multilocularis* vesicles had visibly lost turgidity. The apex was dissected and the remaining fluid removed, ensuring that no protoscoleces were aspirated. EmF was centrifuged (10000 *g*, 10 min) to remove debris, filtered through a 0.2-μm filter and stored at -80℃ until use.

Cell isolation, culture of rat hepatocytes and treatment with EmCM or EmF

Rat hepatocytes were prepared as described previously[28] and cultured in William's E culture medium in a humidified incubator at 37℃ and 5% CO_2 for 20 h before the start of the experiment, supplemented with 100 U/mL penicillin and 100 mg/mL streptomycin (Invitrogen, Paisley, UK), without the addition of hormones or growth factors. During the attachment period (4 h), 2 mmol/L glutamine, 4 mg/mL bovine insulin, 1 μmol/L dexamethasone, and 10% fetal calf serum (Life Technologies Ltd) were added to the medium. Hepatocyte viability was always more than 90% and purity more than 95%.

For the experiment, cells were washed and cultured for 20 h in serum-free insulin-free William's E culture medium, then incubated with either EmF for 15 min, 30 min, 1, 2, 8 and 24 h or EmCM for 15 min, 30 min, 1, 2, 3, 8 and 24 h, respectively.

Western blotting analysis

Western blotting analysis of cell lysates was performed by SDS-PAGE using NuPAGE (Invitrogen, Carlsbad, CA, USA) followed by transfer to nitrocellulose membrane (Invitrogen). Ponceau S (Sigma) staining was used to ensure equal protein loading and electrophoretic transfer. Using the appropriate antibodies, ERK1/2, JNK, p38 and their corresponding phosphoproteins, phosphorylated MAPK/ERK kinase 1/2 (MEK1/2), phosphorylated ribosomal S6 kinase (RSK), phosphorylated transcription factor Elk-1 (Elk-1), [Cell Signaling Technology (Beverly, MA, USA) and β-tubulin (Sigma)] were detected with the WesternBreeze Kit (Invitrogen). The expression levels of p-ERK1/2 /total ERK1/2 (signal at 44 kDa), p-p38/total p38 and p-JNK/total JNK (signal at 54 kDa) proteins (in "relative units") in control cultures and cultures treated with EmCM or EmF were quantified using Quantity One software (Bio-Rad, Hercules, CA, USA).

Assay for cytotoxicity of EmCM or EmF

Primary cultures of rat hepatocytes were plated in 96-multiwell plates. After attachment, they were treated with EmF (diluted by half in William's E culture medium) or pure EmCM for 24 h and cell viability was assessed[29]. No toxic effect was found.

Detection of proliferating cell nuclear antigen (PCNA) in liver sections

Formalin-fixed, paraffin-embedded sections of the five AE patients' livers were stained for the presence of PCNA using a biotinylated anti-PCNA antibody (Boshide Inc., Wuhan, China) according to the manufacturer's instructions. PCNA-positive hepatocytes were counted in three random visual fields of 0.95 mm^2 each, at initial magnification × 20, for each sample, and the number expressed as the percentage of PCNA-positive cells to the total number of cells counted.

Statistics analysis

Data were presented as the mean ± SD and analyzed using SPSS version 11.0 software (SPSS, Chicago, IL, USA). Statistical significance was tested using the Student *t* test; a *P* value of less than 0.05 was considered significant.

RESULTS

ERK1/2 and p38 activation in AE patients

ERK1/2 phosphorylation was assessed in liver samples taken close to and distant from the parasitic lesions in five AE patients. As shown in Figure 1A, ERK1/2 phosphorylation was 1.58-fold to 4.26-fold higher in the liver close to the parasitic lesion than in the distant liver. p38 phosphorylation was found in the liver of all AE patients; it was more prominent in the liver close to the parasitic lesion than in liver distant from the lesion (1.70 to 3.40-fold), except in one patient (0.55-fold) (Figure 1B).

Expression of PCNA in AE patients

The expression of PCNA, an important growth marker and DNA replication regulator, was assessed in the liver close to and distant from the parasitic lesions in five AE patients. As shown in Figure 2A, an increased expression of PCNA was observed in the liver close to the parasitic

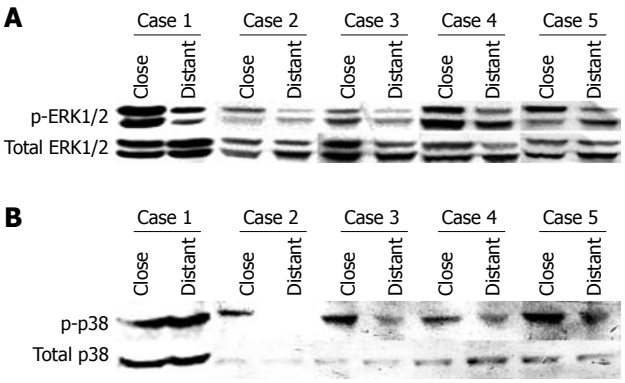

Figure 1 ERK1/2 (A) and p38 (B) activation in liver samples from five AE patients. Western immunoblot analyses were performed on lysates with antibodies that recognize phosphorylated and total ERK1/2 respectively (A), and phosphorylated- and total p38, respectively (B). Close: Liver samples close to the parasitic lesions in AE patients; Distant: Liver samples distant from the parasitic lesions in AE patients.

lesions compared to the liver distant from the parasitic lesion (Figure 2B). Although a faint expression of PCNA could be detected in the distant liver in one case, there was a significant difference between PCNA expression in the hepatocytes close to and distant from the parasitic lesion ($P < 0.05$, Figure 2C).

MAPKs (ERK1/2, JNK and p38) activation by exposure of primary hepatocytes to EmF or EmCM

To investigate whether the MAPKs were also activated in primary cultured hepatocytes in contact with EmF or EmCM, we measured phosphorylated and total ERK1/2, JNK and p38. As shown in Figure 3A, increased ERK1/2 phosphorylation was observed from 15 min to 2 h and peaked at 1 h after incubation with EmF. EmF increased the phosphorylation of ERK1/2 (threonine-202, tyrosine-204) from approximate 2.50-fold at 15 min to 6.50-fold at 1 h (Figure 3B). There was a significant difference between non-treated and EmF-treated liver cell cultures at the 15 min, 30 min and 1 h time-points ($P < 0.05$). In contrast, EmCM only weakly stimulated ERK activity from approximately 1.37-fold at 15 min and approximately 1.84-fold at 8 h to approximately 2.42-fold at 24 h (Figure 3C and D).

EmF slightly activated p38 at 1, 2 and 24 h (Figure 4A). No activation of p38 MAPK could be detected in EmCM-stimulated hepatocytes (Figure 4B).

EmF increased the phosphorylation of JNK (threonine-183, tyrosine-185) from 2.63-fold at 15 min to 2.23-fold at 30 min, respectively (Figure 5A and B). Similar results were found in the EmCM-treated liver cells, as shown in Figure 5C and D: increased JNK phosphorylation was observed from 3.26-fold at 15 min to 1.94-fold at 30 min, respectively, and then there was a decrease to the baseline.

Taken together, these results clearly show that EmF stimulated all 3 classes of MAPKs, but EmCM only induced ERK1/2 and JNK activation in primary hepatocytes.

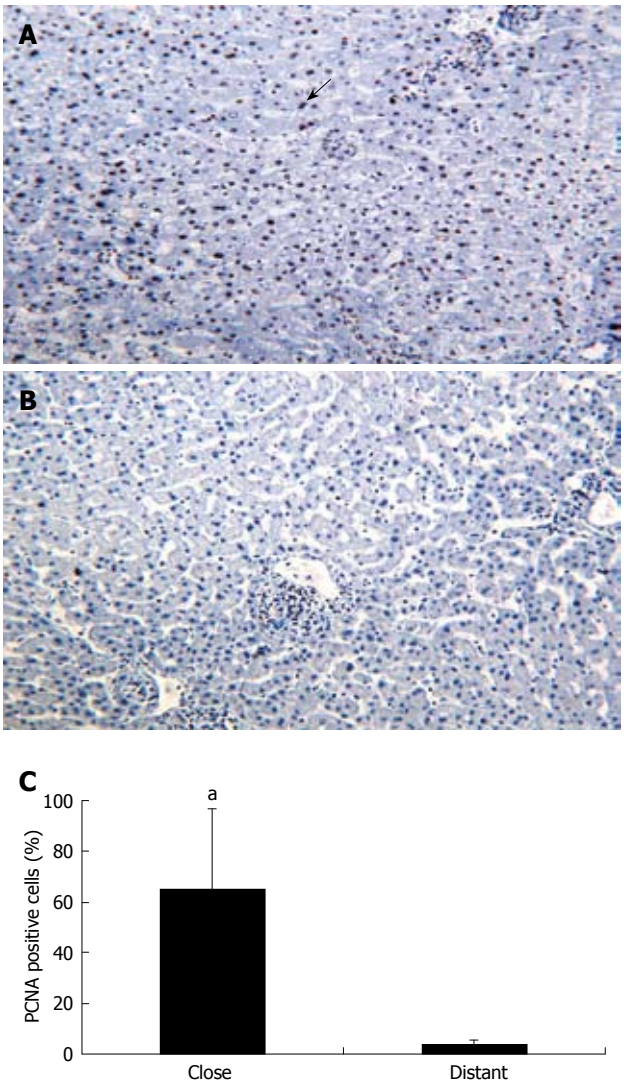

Figure 2 PCNA expression by hepatic cells in the liver from five patients with AE (immunohistochemical analysis). A: Hepatic cells close to the parasitic lesions were strongly labeled by the anti-PCNA antibody; all cells with a dark-brown/black nucleus are positive cells; some of them indicated by an arrow (initial magnification: × 20); B: Hepatic cells distant from the parasitic lesions did not express PCNA (initial magnification: × 20); C: Quantitative expression of PCNA was significantly higher in the liver cells close to the parasitic lesions than in those distant to them ($^aP < 0.05$).

ERK1/2 pathway activation by exposure of primary hepatocytes to EmF or EmCM

To further explore the effect of EmF and EmCM on the ERK activation pathway, we first studied the activation of MEK1/2, the physiological activator of ERK[21,30]. We did indeed observe an activation of MEK1/2 from 15 min to 2 h of EmF exposure (Figure 6A). In contrast, MEK1/2 activation was not detectable at any time points during EmCM exposure (data not shown). Then, we studied the phosphorylation of RSK and Elk-1, cytoplasmic substrates of ERK1/2 and mediators of cell survival[23,31,32]. As shown in Figure 6B, RSK phosphorylation was observed after exposure to EmF and maximal RSK activation was observed at 30 min. No phosphorylation of Elk-1 could be detected neither after EmF nor EmCM incubation (data not shown).

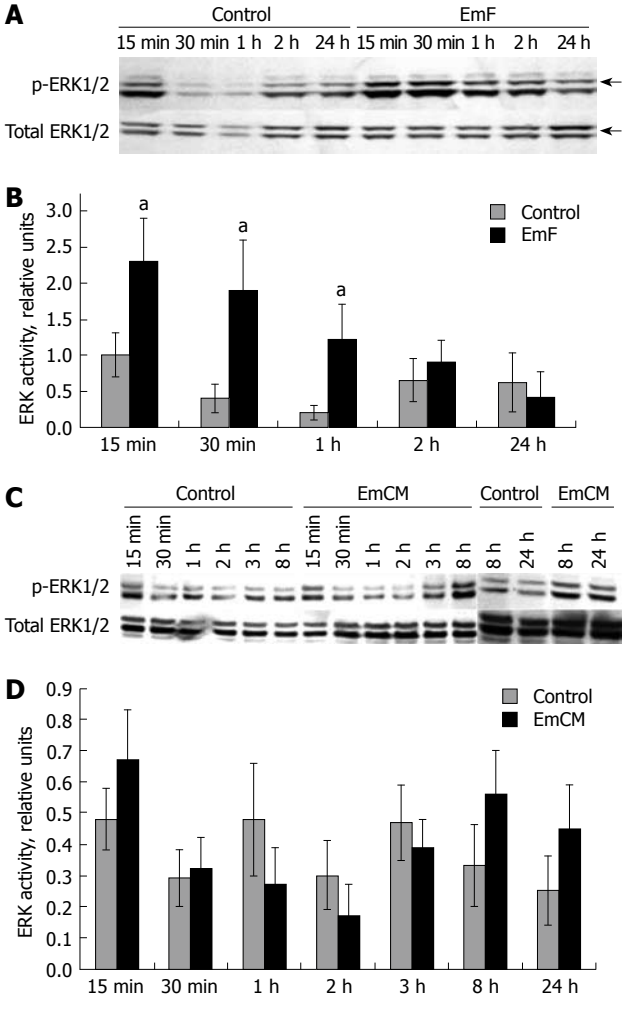

Figure 3 Time course of EmF- or EmCM- induced phosphorylation of ERK1/2 kinase. Primary cultures of rat hepatocytes were stimulated with EmF (A, B) or EmCM (C, D) and harvested at the indicated time points (15 min to 24 h). Western immunoblot analyses were performed on lysates with antibodies that recognize phosphorylated (p-) and total ERK1/2 (A, C), respectively. Relative amount of phosphorylated to total ERK1/2 and ERK1/2 was calculated from semi-quantitative analysis of the Western blotting using densitometry (B, D). $^{a}P < 0.05$, EmF or EmCM-induced *versus* control hepatocytes. All experiments were performed three times independently with similar results.

Thus, EmF exposure, but not EmCM exposure, induced RSK activation in hepatocyte cultures; none of them activated Elk-1.

DISCUSSION

In this study we found a significant influence of *E. multilocularis* metacestode on the activation of MAPK signaling pathways. *In vivo*, in the liver of AE patients, increased proliferation of hepatocytes was observed and ERK1/2 and p38 were phosphorylated, both being higher in the vicinity of the parasitic lesions. *In vitro*, in primary cultures of rat hepatocytes, three MAPKs (p38, JNK and ERK1/2) were activated upon exposure to *E. multilocularis* parasitic fluid, while p38 was undetectable and only JNK was up-regulated after incubation with supernatants of *E. multilocularis* axenic cultures.

The liver has the unique ability to regenerate after injury or loss of tissue. Liver regeneration is controlled

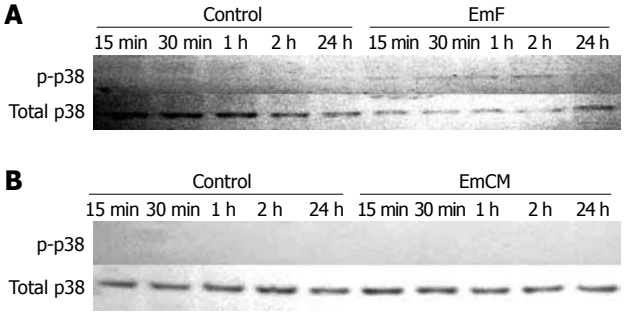

Figure 4 Time course of EmF- or EmCM-induced phosphorylation of p38 kinase. Primary cultures of rat hepatocytes were stimulated with EmF (A) or EmCM (B) and harvested at the indicated time points (15 min to 24 h). Western immunoblot analyses were performed on lysates with antibodies that recognize phosphorylated (p-) and total p38. All experiments were performed three times independently with similar results.

by a wide array of signaling factors and plays a key role in recovery after acute and chronic liver injury[33]. Hepatic cell proliferation represents a central and unique feature of tissue repair after liver injury. ERK1/2 is considered to be an important inducer of the pro-mitogenic pathway and ERK1/2 activation is correlated with hepatocyte DNA replication *in vivo* and hepatocyte proliferation *in vitro*[33,34]. In *E. multilocularis* infection, parasitic influence on liver cell proliferation might be crucial to ensure metacestode survival within the liver. Our data indicate that *E. multilocularis* infection of the liver actually activates ERK1/2 and induces cell proliferation. The major extent of size increase of the normal liver lobes has often been stressed in AE patients[7].

Specific stimulation of hepatocyte proliferation by metacestode-derived substances may add to the regeneration process that normally occurs following liver injury and explain this clinical observation. Such influence may be due either to a direct effect of substances of parasitic origin or to an indirect effect, through a response to host cytokines which are secreted by the macrophages and lymphocytes surrounding the parasitic lesions. A variety of host cytokines are actually present in the periparasitic environment of *E. multilocularis* infection[13]. They include both pro-inflammatory cytokines such as tumor necrosis factor-α, interleukin-6 (IL-6) and IL-1β[13,35] and anti-inflammatory cytokines such as IL-10[36,37] and transforming growth factor-β (TGF-β)[38], and might be responsible for the observed changes in the MAPK system. As *in vivo* studies in infected patients did not allow us to determine precisely the mechanism of activation and the pathways involved, we used *in vitro* cultures of hepatocytes directly in contact with substances of parasitic origin to further analyze the origin of the activation processes.

MAPK activation occurred in rat hepatocyte cultures incubated with fluids of parasitic origin, in the absence of inflammatory cells. We may anticipate that at least part of the activation was related to direct interactions between *E. multilocularis* metacestode-derived components and the liver cells. Cross-functioning between parasite-derived molecules and

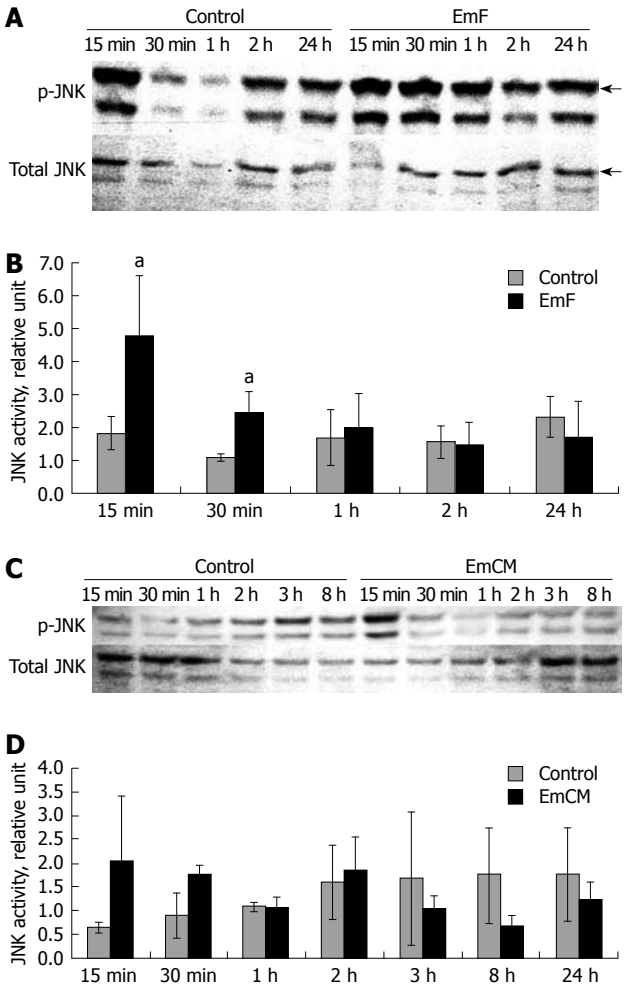

Figure 5 Time course of EmF- or EmCM-induced phosphorylation of JNK. Primary cultures of rat hepatocytes were stimulated with EmF (A, B) or EmCM (C, D) and harvested at the indicated time points (15 min to 24 h). Western immunoblot analyses were performed on lysates with antibodies that recognize phosphorylated (p-) and total JNK respectively (A, C). Relative amount of phosphorylated to total JNK was calculated from semi-quantitative analysis of the Western blots using densitometry (B, D). $^{a}P < 0.05$, EmF or EmCM-induced *versus* control hepatocytes. All experiments were performed three times independently with similar results.

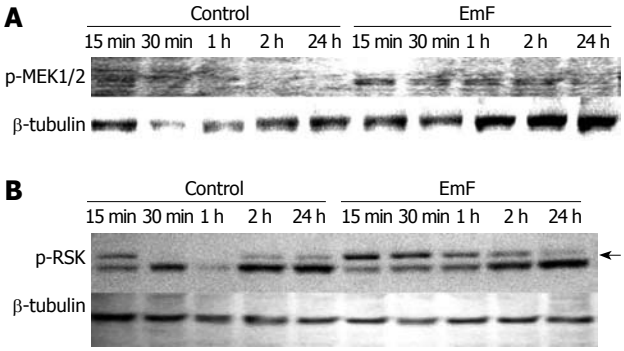

Figure 6 Time course of EmF-induced phosphorylation of MEK1/2 (A) and RSK (B). Primary cultures of rat hepatocytes were stimulated with EmF and harvested at the indicated time points (15 min to 24 h). Western immunoblot analyses were performed on lysates with antibodies that recognize phosphorylated (p-) MEK1/2 and p-RSK. Protein loading control was performed using β-tubulin. MEK: MAPK/ERK kinase.

host liver was described for parasite-derived enzymes: for instance, *E. multilocularis*-derived transglutaminase was shown to efficiently catalyze human liver-derived osteonectin cross-linking[8]. The significant changes observed using EmCM, which is totally free of host components, demonstrated that parasitic components specifically activated JNK and were actually acting on hepatocyte metabolic pathways. The most consistent data, however, were obtained by the incubation of rat hepatic cells with EmF. Upon exposure of hepatic cells to EmF, the expression of phosphorylated ERK1/2 paralleled that of phosphorylated JNKs. EmF exposure also induced the activation of MEK1/2 and RSK in hepatocytes. The differences between both stimuli might result from differences in the concentration of potential activators, EmF being more concentrated than EmCM. Alternatively, they might be due to the simultaneous presence of activating and inhibiting factors after 40 h of metacestode culture, while EmF collected in intermediate hosts infected with *E. multilocularis* for several weeks could be more concentrated in activating factors. Involvement of host factors stored in EmF could also explain the differences. In fact, in addition to proteins secreted by the germinal layer of *Echinococcus* sp. *metacestodes*, the vesicle fluid (often called hydatid fluid) may contain host proteins that are transported across the laminated layer and the germinal layer of the parasite. Albumin and globulins[39], inhibitors of the complement cascade[40] and, recently, host-derived active matrix metalloproteinase 9[41], were found in *Echinococcus granulosus* hydatid fluid or bound to the cyst wall. Heat shock proteins hsp70 and hsp20, which can interfere with MAPKs, especially p38, were also found in *E. granulosus* hydatid fluid[39]. It is highly likely that hydatid fluid from both species, *E. granulosus* and *E. multilocularis*, may also contain cytokines and growth factors of host origin and serve as storage for continuous release of factors both to the parasite and to the host through the laminated layer which appears critical at the host-parasite interface[42]. Dual interactions could thus ensure growth and survival of the parasite while interfering with host liver cells.

Several lines of evidence suggest that *E. multilocularis* differentiation is dependent on the receipt of appropriate host signals through surface receptors and their transduction through functional MAPK signaling pathways in the parasite[16,18,43,44]. Our data show that the reverse, i.e. parasite-derived signals efficiently acting on MAPK signaling pathways in host liver cells, is actually operating. Although the precise nature of these signals cannot be inferred from the present study, insulin and EGF, which have been identified as candidates for MAPK activation from the host to the parasite[18,44] are possible candidates for MAPK activation from the parasite to the host. This has to be studied by additional experiments *in vitro*. In addition, other candidates cannot be ruled out; among them, TGF-β, which is present in the serum of infected experimental intermediate hosts[45] and in the periparasitic environment of *E. multilocularis* in the human liver[38]. TGF-β does not activate the MAPKs directly but may exert an indirect influence through

the activation of Smads. *E. multilocularis* metacestode is sensitive to TGF-β signaling[46,47] and the metacestode ERK-like kinase, EmMPK1, phosphorylates EmSmadD, a metacestode analogue of the Co-Smads of the TGF-β signaling cascade[46]. TGF-β is involved in immune suppression/tolerance[48], liver cell proliferation[49] and liver fibrosis, where it plays a major role in the activation and progression processes[50], where all three effects are essential to the pathogenesis of AE. This does not preclude, however, the importance of other cytokines or stress molecules.

In summary, three MAPKs, p38, JNK and ERK1/2, and the upstream (MEK1/2) and downstream (RSK) components of the ERK1/2 signaling pathways, are activated in primary cultures of rat hepatocytes by parasite- and/or host-derived substances. JNK activation by host-free supernatant of *E. multilocularis* cultures suggests that liver cell signaling pathways are actually activated by parasitic components. Hepatic proliferation in AE could thus be induced through a direct influence of the parasite and not only linked to the usual reaction of hepatic cells to the occupying process that takes place in the liver. The current investigation is the first which addresses the possible influence of *E. multilocularis*-related molecules on liver cells and demonstrates changes that are consistent with liver cell signaling through these molecules. Attempts to elucidate the nature and origin of the parasite-derived factors which influence intracellular signaling pathways in host cells may especially clarify the mechanism used by *E. multilocularis* to increase cell proliferation but also concomitant events, including parasite survival, immune suppression and induction of liver fibrosis.

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COMMENTS

Background

Changes in the metabolic pathways involved in homeostasis and growth regulation of hepatic cells, and especially in the mitogen-activated protein kinase (MAPK) system, have been extensively studied in infectious/inflammatory conditions. Very little is known, however, on the capacity of helminth parasites and/or their components/secretions to influence liver cell homeostasis metabolic pathways and no study has reported on the activation pattern of liver cell MAPK during *Echinococcus multilocularis* (*E. multilocularis*) infection. Helminths developing in the liver may influence hepatic cell proliferation through the activation of MAPKs. The authors thus explored the effect of *E. multilocularis* on the activation of MAPKs signaling pathways and on liver cell proliferation.

Research frontiers

MAPKs play important roles in signal transduction from the cell membrane to the nuclear transcriptional factors; they cross-communicate and regulate the balance between cell survival and cell death in acute and chronic liver injury. The dynamic balance of their activities appears critical in acute liver injury such as viral hepatitis, drug- or toxin-induced toxicity or acute rejection after liver transplantation, as well as in chronic liver injury. Thus, exploring this system is the best way to study the interactions between the parasite and the host, relating to proliferation processes.

Innovations and breakthroughs

It is the first *in vivo* demonstration that a helminth parasite influences the proliferation/regeneration of hepatic cells and the concomitant activation of the MAPK metabolic pathway. Previous studies have demonstrated an influence of the host liver on the MAPK cascade in *E. multilocularis* metacestode; the data suggest that the reverse, i.e. parasite-derived signals efficiently acting on MAPK signaling pathways in host liver cells, is actually operating.

Applications

The observed changes could be involved in the development of the massive hepatomegaly often observed as a presenting symptom in alveolar echinococcosis in humans, and which makes major hepatic resections a therapeutic option for this disease. It could also be involved in other aspects of the host-parasite relationship, including parasite survival, immune suppression and induction of liver fibrosis. This opens new avenues of research to understand parasite-host interactions in the liver.

Terminology

MAPKs are cell signaling pathways that include c-Jun N-terminal kinase (JNK), p38 MAPK and extracellular signal-regulated kinase (ERK). Generally, the JNK and p38 MAPK families appear to be pro-apoptotic, while the ERK pathway appears to be anti-apoptotic in specifically mediating cell growth and survival signals in many cell types. The metacestode of *E. multilocularis* is the larval form of this cestode, which develops in rodent intermediate hosts and is responsible for the hepatic disease alveolar echinococcosis in humans.

Peer review

The manuscript describes an investigation on cell signaling events in the liver induced by infection with *E. multilocularis*. Experiments were performed on samples of infected human liver specimens or using conditioned media or vesicle fluid from infected animals to induce activation of the MAPK pathway in cultured hepatocytes. Whilst the *in vitro* hepatocyte data are supported by evidence of global MAPK activation in whole liver lysates, it would be interesting to complete the study by immunostaining with phospho-specific monoclonal antibodies for ERK and p38 in liver tissue, to identify which cell types are being modulated by the presence of parasite and the precise location of these cells. The study is well conceived and on the whole the experiments have been well thought out.

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C-type natriuretic-peptide-potentiated relaxation response of gastric smooth muscle in streptozotocin-induced diabetic rats

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Abstract

AIM: To study the sensitivity of gastric smooth muscle to C-type natriuretic peptide (CNP) in streptozotocin (STZ)-induced diabetic rats.

METHODS: The spontaneous contraction of a gastric smooth muscle strip was recorded by using physiological methods in rats. The expressions of CNP and natriuretic peptide receptor-B (NPR-B) in gastric tissue were examined by using immunohistochemistry techniques in the diabetic rat.

RESULTS: At 4 wk after injection of STZ and vehicle, the frequency of spontaneous contraction of gastric smooth muscle was significantly reduced in diabetic rats, and the frequency was decreased from 3.10 ± 0.14 cycle/min in controls to 2.23 ± 0.13 cycle/min (n = 8, P < 0.01). However, the amplitude of spontaneous contraction was not significant different from the normal rat. CNP significantly inhibited spontaneous contraction of gastric smooth muscle in normal and diabetic rats, but the inhibitory effect was significantly potentiated in the diabetic rats. The amplitudes of spontaneous contraction were suppressed by 75.15% ± 0.71% and 58.92% ± 1.32% while the frequencies were decreased by 53.33% ± 2.03% and 26.95% ± 2.82% in diabetic and normal rats, respectively (n = 8, P < 0.01). The expression of CNP in gastric tissue was not changed in diabetic rats, however the expression of NPR-B was significantly increased in diabetic rats, and the staining indexes of NPR-B were 30.67 ± 1.59 and 17.63 ± 1.49 in diabetic and normal rat, respectively (n = 8, P < 0.01).

CONCLUSION: The results suggest that CNP induced an inhibitory effect on spontaneous contraction of gastric smooth muscle, potentiated in diabetic rat *via* up-regulation of the natriuretic peptides-NPR-B-particulate guanylyl cyclase-cyclic GMP signal pathway.

Key words: Diabetes; Natriuretic peptide receptor type B; Gastric smooth muscle; Gastroparesis; Spontaneous contraction

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INTRODUCTION

Gastroparesis (delayed gastric emptying) is frequent in diabetic patients. It is a well-recognized complication of long-standing diabetes. The symptom complex typically associated with gastroparesis occurs in 25%-55% of patients with long-standing type 1 or type 2 diabetes[1,2]. Symptoms of diabetic gastropathy can range from mild dyspepsia to recurrent vomiting and abdominal pain, and may progress to irreversible end-stage gastric failure known as gastroparesis. Gastroparesis seriously affects the quality of life. There is deterioration in glycemic control and incapacitating symptoms such as malnutrition, water and electrolyte imbalance, and aspiration may occur. However, the pathophysiology of diabetic gastropathy

and gastroparesis, including impaired fundic and pyloric relaxation and impaired electrical pacemaking, is still not delineated[3,4]. It is generally considered that diabetic gastropathy and gastroparesis may be due to visceral autonomic neuropathy, hyperglycemia and degeneration of smooth muscle. Several physiological studies have reported that dysfunction of gastric smooth muscle in diabetes is associated not only with neural factors, but also with intracellular signaling pathways[5,6].

Since atrial natriuretic peptide (ANP) was isolated from atrium by de Bold *et al*[7] in 1981, brain natriuretic peptide (BNP), C-type natriuretic peptide (CNP), dendroaspis natriuretic peptide (DNP), micrurus natriuretic peptide (MNP), and ventricular natriuretic peptide (VNP) were found in succession. Natriuretic peptides (NPs) are distributed all over the body besides the heart and exert natriuretic-diuretic, vasorelaxation, and other functions designed to decrease blood pressure and to control electrolyte homeostasis. Three types of single-transmembrane natriuretic peptide receptors (NPRs) for ANP, BNP and CNP have been identified[8,9]; i.e. NPR type A (NPR-A), type B (NPR-B) and type C (NPR-C). NPR-A and NPR-B receptors have membrane-bound particulate guanylate cyclase (pGC), which catalyzes the formation of cGMP from GTP[10-12]. NPR-A preferentially binds ANP and BNP, but has a low affinity for CNP; NPR-B has a much higher affinity for CNP than either ANP or BNP[13]. NPs are also secreted from gastric mucosa[14-16]. Our previous study indicated that CNP relaxes gastric circular and longitudinal smooth muscles in human, rat and guinea-pig stomach, and that NPRs are distributed in rat gastric smooth muscle layer[17-19]. In smooth muscle, CNP activates its cognate NPR-B, which includes an intracellular pGC domain and catalyzes the synthesis of cGMP within the cytosol[20]. CNP and NPR-B have been detected in the stomach[17,21,22]. CNP mRNA expression was increased in the kidney of streptozotocin (STZ)-induced diabetic rats and NPR-B expression was enhanced in vascular smooth muscle in the diabetic mouse[23,24].

However, it is not clear what the relationship is between diabetic gastroparesis and the natriuretic peptide signal pathway. In the present study, the possibility as to whether the natriuretic peptide-dependent cGMP signal pathway is involved in diabetic gastropathy or gastroparesis was investigated in STZ-induced rats.

MATERIALS AND METHODS

STZ-induced diabetic animal model

Male Sprague-Dawley rats (200-220 g) were purchased from the Experimental Animal Center of Yanbian University College of Medicine. Animals were allowed to have free access to food and water. A total of 30 rats were divided into two groups (15 per group): one was the normal control group and another was the diabetic group. All rats were used for the experiment at 4 wk after the injection of STZ and vehicle. Diabetes was induced by a single intraperitoneal injection of STZ (Sigma-Aldrich, St. Louis, MO, USA) in 0.1 mol/L citrate buffer (pH 4.0) at a dose of 65 mg/kg body weight[6]. Control animals received an equal volume of citrate buffer. The glucose concentration in tail-blood was determined at the end of the experiment with a SureStepPlus apparatus (LifeScan, Milpitas, CA, USA). Diabetes was confirmed by measurement of blood glucose concentrations and defined as blood glucose above 350 mg/dL. Animals were treated in accordance with the Guide for Care and Use of Laboratory Animals published by the National Institutes of Health (China).

Organ bath study

Four weeks after treatment with STZ and vehicle, animals were anesthetized with sodium pentobarbital (50 mg/kg, ip) and then the abdomen was opened. The stomach was removed and placed in pre-oxygenated Kreb's Ringer solution at room temperature. The mucosal layer was removed and the strips (about 2.0 mm × 15.0 mm) of gastric antral circular muscle from control and diabetic rats were prepared, respectively. The longer axis of the stomach was cut parallel to the circular muscle fibers. Muscle strips were placed in a 2-mL organ bath containing modified Kreb's Ringer solution at 37℃, aerated with 95% O_2 and 5% CO_2. One end of the muscle strip was anchored to a stationary support, and the other end was connected to an isometric force transducer (Grass FT03C, Quincy, MA, USA). The tension loaded onto each strip was 1.0 g. Isometric contractions were recorded using a computerized data acquisition system (Power Lab/8SP, AD Instruments, Castle Hill, NSW, Australia). The muscle strip was allowed to incubate for at least 40 min before experiments were started. The composition of the modified Kreb's Ringer solution (mmol/L) was as follows: NaCl 120; KCl 4.7; $CaCl_2$ 2.0; $MgCl_2$ 1.2; $NaHCO_3$ 25; KH_2PO_4 1.2; and glucose 14.

Immunohistochemistry study

Tissues of normal control and STZ-diabetic rats stomach antrum were fixed in 4% buffered formalin for 24 h, dehydrated in ethanol, and embedded in paraffin. Sections were cut at 5 μm, and mounted on poly-L-lysine-coated slides. Sections were deparaffinized in three changes of xylene, hydrated in a graded ethanol series, and washed in tap water. Endogenous peroxidase activity was blocked by immersing slides in 0.3% H_2O_2 for 30 min. After being washed in phosphate buffered saline (PBS), slides were incubated for 45 min at 37℃ in a humidified container with normal goat serum to block non-specific binding of the primary antibody. The blocking serum was removed by gentle tapping, and slides were incubated for 24 h at 4℃ in a humidified container with either rabbit anti-CNP (1:600, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or rabbit anti-NPR-B (1:500, Santa Cruz Biotechnology). After being washed thoroughly in PBS, slides were incubated for 30 min at 37℃ in a humidified container with biotin-labeled goat anti-rabbit serum. After being washed in PBS, the peroxidase-

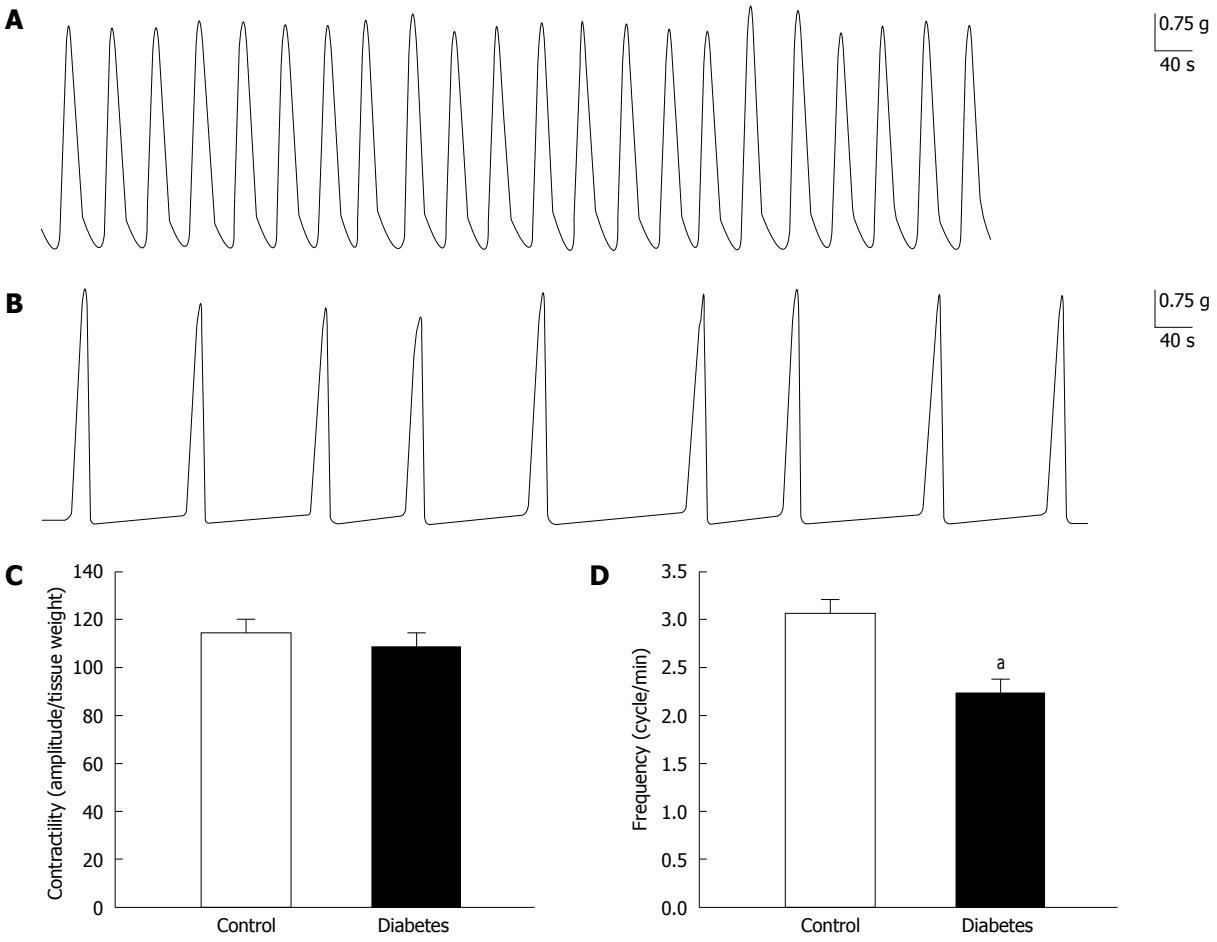

Figure 1 Comparison of gastric smooth muscle contractilities between normal and diabetic rats. A, B: The row traces gastric smooth muscle spontaneous contractions in normal and diabetic rats; C, D: Summary of the contractility in normal and diabetic rats. The contractility per weight of gastric smooth muscle strip was not significantly different between normal and diabetic rats (A-C, $n = 8$, $P > 0.05$). However, the frequency of spontaneous contraction was significantly depressed in diabetic rats (A, B and D, $n = 8$, $^{a}P < 0.01$).

labeled streptavidin complex reagent was added, and the slides were incubated for 30 min at 37℃ in a humidified container. After being washed in PBS, antibody binding was visualized using 3,3'-diaminobenzidine. Slides were washed in running tap water, counterstained lightly with hematoxylin, and mounted in permount. For negative controls, sections were incubated with PBS in place of the primary antibody.

Drugs

CNP (rat CNP-22), STZ, cGMP antibody and chemicals were purchased from Sigma-Aldrich (St. Louis, MO, US). CNP was dissolved in distilled water (1 mmol/L) and further diluted in the superfusion buffer to the concentrations indicated in the text.

Statistics analysis

The staining index was calculated from the staining intensity and area by means of image analysis software, in three areas per section, three sections per group, and weak, medium and strong CNP and NPR-B staining intensities graded as 1, 2 and 3 points according to Feng J Lai's method[25]. The contractility = amplitude of spontaneous contraction (g)/gastric smooth muscle strip weight (g). Inhibitory percentages = amplitude in control - amplitude decreased by CNP/amplitude in control × 100%. Staining index = staining intensity × staining area. Data were expressed as mean ± SE. Statistical significance was evaluated by *t* test. Differences were considered significant when $P < 0.05$.

RESULTS

Change in body weight and plasma glucose

Rats were used for experiments at 4 wk after injection with STZ. At the time of the experiment, all STZ-treated rats exhibited hyperglycemia; their blood glucose concentrations (478.0 ± 27.9 mg/dL) were significantly higher than those of the non-diabetic control rats (108.9 ± 11.4 mg/dL, $n = 8$, $P < 0.001$) and the body weights of the diabetic rats (209.7 ± 8.0 g) were significantly lower than those of the control rats (247.4 ± 13.1 g, $n = 8$, $P < 0.05$).

The spontaneous contraction of gastric smooth muscle

To determine the extent of gastric motility impediment in diabetic rats the spontaneous contractions of gastric smooth muscle strips were observed in control and

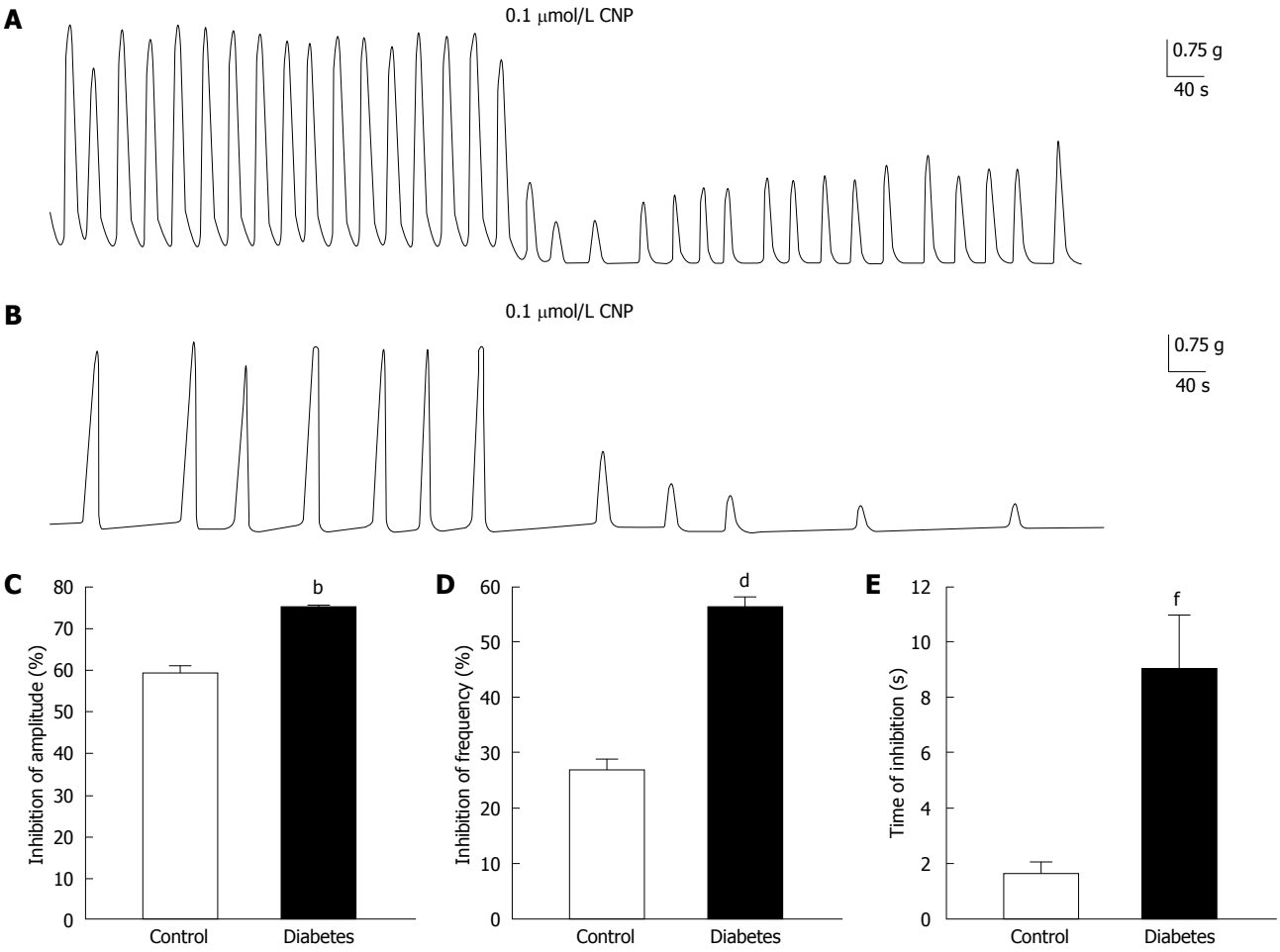

Figure 2 The sensitivity of gastric smooth muscle to CNP. A, B: The row traces gastric smooth muscle spontaneous contractions in response to CNP in normal and diabetic rats; C-E: Summary of the contractility in response to CNP in normal and diabetic rats. CNP induced relaxation of gastric antral smooth muscle in control and diabetic rats (A, B). However, CNP-induced inhibition of spontaneous contraction was potentiated in diabetic rats, and the amplitude (C, $n = 8$, $^{b}P < 0.01$) and frequency (D, $n = 8$, $^{d}P < 0.01$) of spontaneous contraction were more potentially suppressed by CNP in diabetic rats. The inhibition time of CNP of spontaneous contraction was significantly prolonged in diabetic rats (E, $n = 8$, $^{f}P < 0.01$).

diabetic rats. At 4 wk after injection of STZ and vehicle, the spontaneous contraction was recorded in gastric smooth muscle strips of normal and diabetic rats. In order to compare the contractilities of gastric smooth muscle between normal and diabetic rats, the amplitudes of spontaneous contraction of gastric smooth muscle were normalized by every muscle strip weight. The frequency of spontaneous contraction was significantly decreased in diabetic rats, while the amplitude of spontaneous contraction was not significantly affected in diabetic rats (Figure 1A and B). The frequency of spontaneous contraction was decreased from 3.10 ± 0.14 cycle/min of the control to 2.23 ± 0.13 cycle/min (Figure 1D, $n = 8$, $P < 0.01$), however, the contractilities were 115.18 ± 8.69 and 109.34 ± 6.54 in normal and diabetic rats, respectively (Figure 1C, $n = 8$, $P > 0.05$).

The sensitivity of gastric smooth muscle to CNP

To determine the role of the natriuretic peptide signal pathway in diabetic gastroparesis, the effect of CNP on spontaneous contraction was observed in normal and diabetic rats. CNP significantly inhibited the spontaneous contractions in both groups (Figure 2A and B), however, the inhibitory effect was potentiated in diabetic rats. The amplitude of spontaneous contraction was suppressed by 58.92% ± 1.32% and 75.15% ± 0.71% in normal and diabetic rats, respectively (Figure 2C, $n = 8$, $P < 0.01$). The frequency of spontaneous contraction was decreased by 26.95% ± 2.82% and 53.33% ± 2.03% in normal and diabetic rats, respectively (Figure 2D, $n = 8$, $P < 0.01$). The time of CNP-induced inhibition (inhibition time) was measured as the time from starting to reduce the amplitude of spontaneous contraction to starting to recover from peak inhibition. The inhibition time was prolonged from 1.43 ± 0.80 min of control to 8.95 ± 2.07 min in diabetic rats (Figure 2E, $n = 8$, $P < 0.01$).

CNP and NPR-B expression in gastric tissues

Since the CNP-induced inhibition of spontaneous contraction was potentiated in diabetic rats, the expressions of CNP and NPR-B in gastric tissues were further confirmed. There was no CNP immunopositive expression in negative controls of normal and diabetic rats (Figure 3A and B). The CNP immunopositive brown granules were mainly expressed in gastric muscle layers of normal and diabetic rats (Figure 3C and D),

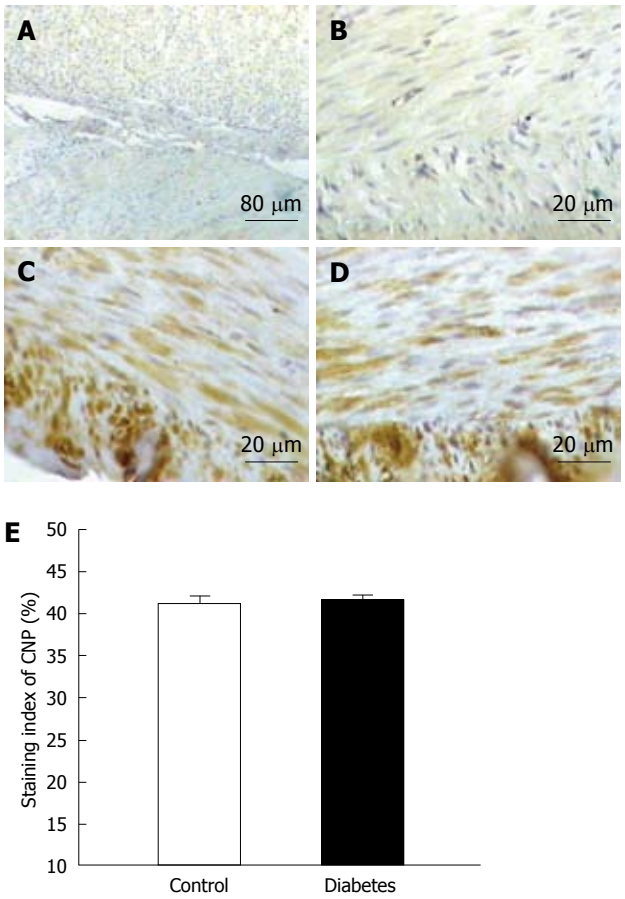

Figure 3 CNP expression in gastric tissues in normal and diabetic rats. A-D: CNP expression in gastric smooth muscle in normal and diabetic rats. In negative controls CNP was not expressed in normal and diabetic rats (A, B) and the CNP immunopositive brown granules were mainly expressed in gastric muscle layers of normal and diabetic rats (C, D); E: Summary of CNP expression in normal and diabetic rats. The staining indexes were not significantly different between normal and diabetic rats (E, n = 8, P > 0.05). Scale bars = 80 μm (A), 20 μm (B-D).

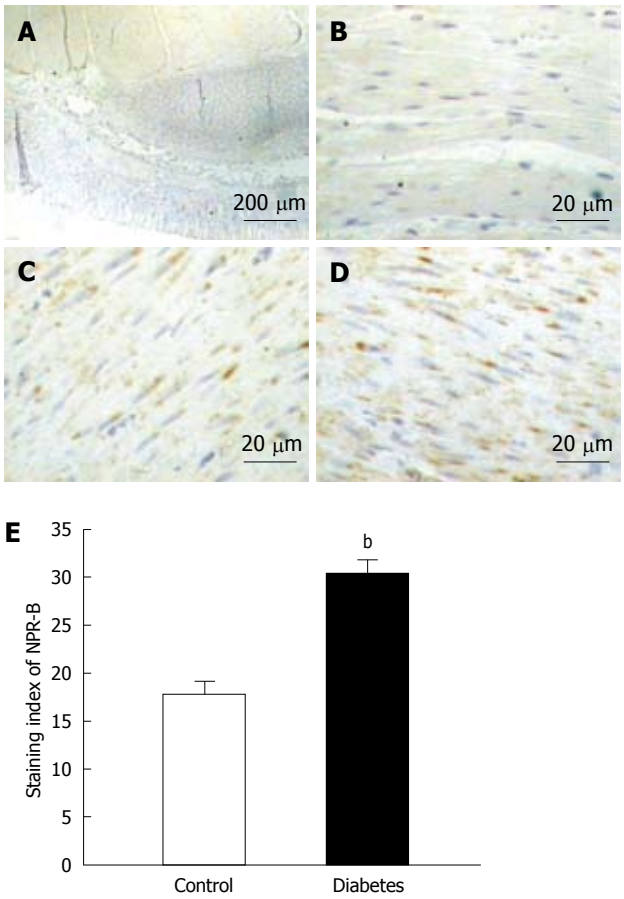

Figure 4 NPR-B expression in gastric tissues in normal and diabetic rats. A-D: NPR-B expression in gastric smooth muscle in normal and diabetic rats. There was no NPR-B immunopositive expression in negative controls of normal and diabetic rats (A, B). The NPR-B immunopositive brown granules were expressed in gastric antral smooth muscle in normal and diabetic rats. The staining was deeper in diabetic rats (C, D); E: Summary of NPR-B expression in normal and diabetic rats. The staining indexes were increased significantly in diabetic rats (E, n = 8, ^bP < 0.01). Scale bars = 200 μm (A), 20 μm (B-D).

and the staining indexes were not significantly different between normal and diabetic rats (Figure 3E, n = 8, P > 0.05). There was no NPR-B immunopositive expression in negative controls of normal and diabetic rats (Figure 4A and B). The NPR-B immunopositive brown granules were expressed in gastric antral smooth muscle in normal and diabetic rats, however the staining was deeper in diabetic rats (Figure 4C and D). The staining indexes were increased from 17.63 ± 1.49 in controls to 30.67 ± 1.59 in diabetic rats, and there were significant differences between normal and diabetic rats (Figure 4E, n = 8, P < 0.01).

DISCUSSION

The effects of CNP on gastrointestinal motility have been described by some reports: relaxant effect on chick rectum muscle strip[26] and guinea pig cecum circular smooth muscle[27], and inhibitory effect on rabbit colon[28]. We previously reported that CNP significantly inhibited spontaneous contraction of gastric smooth muscles in rats, guinea pigs and humans[17]. Although previous studies demonstrated that spontaneous activity of the smooth muscle in the gastrointestinal tract was attenuated in diabetic-model animals[29-31], no studies were made of the relationship with the NPR-pGC-cGMP signal pathway. In our present study, at 4 wk after injection of STZ and vehicle, the frequency of spontaneous contraction was significantly depressed in diabetic rats (Figure 1A and B), while the amplitude of spontaneous contraction was not significantly affected in diabetic rats (Figure 1C). CNP induced relaxation of gastric antral circular smooth muscle in normal and diabetic rats, however the relaxation response induced by CNP was significantly potentiated in diabetic rats (Figure 2). The results indicate that the gastric smooth muscles were more sensitive to CNP in the diabetic rats than in the normal rats, and they suggest that the NPs-NPR-B-pGC-cGMP signal pathway may be upregulated in STZ-induced diabetic rat.

Three types of single-transmembrane NPRs for ANP, BNP and CNP have been identified[8,9], i.e. NPR-A, NPR-B and NPR-C. NPR-A and NPR-B have membrane-bound pGC which catalyzes the formation of cGMP from GTP[10-12]. NPR-A preferentially binds ANP and BNP, but has a low affinity for CNP, NPR-B

has a much higher affinity for CNP than either ANP or BNP[13]. CNP mRNA expression was increased in the kidney of STZ-induced diabetic rats and NPR-B expression was enhanced in vascular smooth muscle in the diabetic mouse[23,24].

In smooth muscle, CNP generally causes relaxation by eliciting membrane-bound pGC-mediated cGMP production[32]. Moreover, many experiments also demonstrated that CNP cognate receptors were distributed in gastrointestinal smooth muscle[23,24,28]. In our present study the NPR-B immunopositive brown granules were increased in the gastric antral smooth muscle of diabetic rats (Figure 4). However, the CNP expression in gastric muscle was not significantly different from normal rats (Figure 3). These results suggest that the NPs-NPR-B-pGC-cGMP signal pathway may be involved in diabetic gastropathy *via* increasing of the NPR-B expression. Furthermore, the data are compatible with the idea that up-regulation of the NPs-NPR-B-pGC-cGMP signal pathway may be an important factor which hastens or induces the disorder of gastric motility, and occurs concomitantly with development of gastrointestinal dysfunction, for example, gastroparesis. Thus, every stage of the NPs-NPR-B-pGC-cGMP signal pathway may be a potential target for investigating the mechanism of diabetic gastropathy or gastroparesis and preventing diabetic gastrointestinal dysfunction.

In summary, this study has demonstrated that diabetes firstly induces frequency depression of gastric motility but not contractility. The CNP-induced relaxation response is potentiated in STZ-induced diabetic rats, and this is related to increased NPR-B expression in the gastric smooth muscle. These results suggest that the NPs-NPR-B-pGC-cGMP signal pathway plays an important role in diabetic gastropathy or gastroparesis.

COMMENTS

Background

A common gastrointestinal complication of diabetes is gastroparesis. However, the pathogenesis is not clear yet. A recent study has indicated that atrial natriuretic peptide (ANP) is secreted from gastric mucosa and plays an inhibitory role in the regulation of gastrointestinal motility, but the effect of the natriuretic peptides (NPs) signal pathway on diabetic gastroparesis has not been reported.

Research frontiers

NPs are distributed all over the body besides the heart, for example, the gastrointestinal tract and enterochromaffin cells in gastrointestinal mucosa secrete NPs. However, the many functions of NPs in the gastrointestinal tract in physiological and pathophysiological conditions need to be explored. In the present study, the possibility as to whether the NPs/cGMP signal pathway is involved in diabetic gastroparesis was investigated in streptozotocin-induced diabetic rats.

Innovations and breakthroughs

Recent reports have highlighted the pathogenesis of diabetic gastroparesis. This is the first study to report that the expression of NP receptor type B in gastric tissue is increased and the sensitivity of gastric smooth muscle to C-type NP (CNP) is significantly enhanced in the diabetic rat. This study suggests that the NPs/cGMP signal pathway may be involved in diabetic gastroparesis.

Applications

By understanding that the NPs/cGMP signal pathway may be involved in diabetic gastroparesis, this study may represent a future strategy for therapeutic or preventive intervention in the treatment of patients with diabetes.

Terminology

Gastroparesis (delayed gastric emptying) is frequent in diabetic patients. Symptoms of diabetic gastropathy can range from mild dyspepsia to recurrent vomiting, abdominal pain and may progress to gastric failure known as gastroparesis. NPRs are natriuretic peptide receptors for ANP, brain natriuretic peptide and CNP.

Peer review

It is an interesting article pointing to a novel mechanism that may explain diabetic changes in gastric function. The results showed are logical, attractive and congruent. In many ways the work is interesting and quite novel and is probably worthy of publication.

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BRIEF ARTICLES

Hyperferritinemia is a risk factor for steatosis in chronic liver disease

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Abstract

AIM: To investigate the relationship between ferritin and steatosis in patients with chronically abnormal liver function tests (LFTs) and high ferritin level.

METHODS: One hundred and twenty-four consecutive patients with hyperferritinemia (male > 300 ng/mL, female > 200 ng/mL) were evaluated; clinical, biochemical and serological data, iron status parameters, *HFE* gene mutations and homeostasis model assessment score were obtained. Steatosis was graded by ultrasound as absent or present. Histology was available in 53 patients only.

RESULTS: Mean level of ferritin was 881 ± 77 ng/mL in men and 549 ± 82 ng/mL in women. The diagnosis was chronic hepatitis C in 53 (42.7%), non-alcoholic fatty liver disease/non-alcoholic steatohepatitis in 57 (45.9%), and cryptogenic liver damage in 14 (11.3%). None was diagnosed as hereditary hemochromatosis (HH). Hepatic siderosis on liver biopsy was present in 17 of 54 (32%) patients; grade 1 in eight and grade 2 in nine. Overall, 92 patients (74.2%) had steatosis. By logistic regression, ferritin and γ-glutamyltransferase were independent predictors of steatosis. Ferritin levels were significantly related to low platelet count, steatosis and hepatitis C virus infection.

CONCLUSION: In a non-obese cohort of non-alcoholic patients with chronically abnormal LFTs without HH, high serum ferritin level is a risk factor for steatosis.

Key words: Steatosis; Serum ferritin; Chronic liver disease; Hepatitis C; γ-glutamyltransferase

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INTRODUCTION

There may be high serum ferritin levels in systemic inflammatory conditions and in renal, liver and neoplastic diseases[1,2]. Among patients with chronic liver disease, high serum ferritin, besides being a hallmark of hereditary hemochromatosis (HH), is frequently found in chronic hepatitis C, in alcoholic or non-alcoholic steatohepatitis (NASH), and in non-alcoholic fatty liver disease (NAFLD).

A raised ferritin level, with an increased transferrin saturation and liver iron concentration, is a typical presentation of HH, an autosomal recessive disorder linked to *HFE* gene mutations[3], which account for most cases of HH in northern Europe and the USA[4]. Epidemiological studies in Mediterranean populations have shown that C282Y occurs only sporadically, while H63D is found among 13.5% of the general population[5]. In this area, *HFE* polymorphism seems to have a modest diagnostic relevance, since many cases of HH do not display the classic pattern of mutations[5]. It

has been suggested that *HFE* mutations may be involved in cases of liver disease complicated by iron overload and in patients with type 2 diabetes[6].

Patients with chronic hepatitis C virus (HCV) infection often have elevated serum iron indices[7], but these do not reflect accurately hepatic iron content, nor are they able to predict clinically important endpoints, such as progression of fibrosis and responsiveness to interferon-based regimens[8-10]. Studies attempting to link iron and the course of chronic hepatitis C have been inconclusive[11]. In chronic hepatitis C, steatosis is a common histological finding and occurs in 30%-70% of patients[12-13]. The biological mechanism underlying steatosis in HCV infection is not definitively understood and is considered to be multifactorial with metabolic mechanisms, including insulin resistance (IR)[14] and iron overload[8,11,15]. In fact, steatosis in patients infected by HCV genotype 1 is linked to a raised γ-glutamyltransferase (GGT) and to IR as a result of lipid peroxidation in the liver[14]. The high prevalence of diabetes in subjects chronically infected with HCV has been ascribed to an increase in IR mediated by an increase in iron deposits[16,17].

In NAFLD, recent studies[18-20] have reported conflicting data on the role of iron in causing liver damage. George *et al*[21] and Bonkovsky *et al*[22] have shown that patients with NAFLD and iron overload have more severe liver disease, whereas Younossi *et al*[19] and Angulo *et al*[20] did not observe any relationship between iron and clinical or pathological outcomes in patients with NAFLD. Mendler *et al*[23] have reported that patients with NAFLD have no more iron overload than patients with isolated steatosis, and that the *HFE* genotype does not influence liver damage, although unexplained hepatic iron overload is nearly always associated with metabolic abnormalities.

We analyzed in a cross-sectional study a cohort of non-obese, non-alcoholic patients with compensated chronic liver disease characterized by elevated serum ferritin levels, of varying etiology, excluding HH, to reassess the link between hyperferritinemia and other markers of the metabolic syndrome, mainly steatosis.

MATERIALS AND METHODS

Patients

We studied all patients consecutively referred to our Gastroenterology & Hepatology Unit, a tertiary referral center, between January 2001 and January 2004. Patients were included in the study if they had abnormal liver function tests and a high serum ferritin level, and if their clinical workup conclusively excluded a final diagnosis of HH. HH was excluded by measurement of transferrin saturation following an overnight fast, according to American Association for the Study of Liver Diseases practice guidelines[24].

Serum ferritin was considered raised according to the WHO criteria if > 300 ng/mL in men and > 200 ng/mL in women. Patients were excluded if they had a history of alcohol abuse (alcohol consumption > 30 g/d in men and > 20 g/d in women), obesity [body mass index (BMI) $\geqslant$ 30], transferrin saturation > 45%, hepatitis B surface antigen positivity, autoimmune hepatitis, celiac disease, Wilson disease, α-1-antitripsin deficiency, porphyria cutanea tarda, or previous antiviral treatment in patients with chronic HCV infection. Alcohol intake and drug use or abuse was evaluated through the administration of a questionnaire. Concomitant inflammatory diseases potentially capable of causing hyperferritinemia were ruled out on the basis of the absence of clinical signs or abnormal blood test results (erythrocyte sedimentation rate, rheumatoid factor, and C reactive protein).

One hundred and twenty-four consecutive patients fitting the above criteria were selected from about 1800 subjects admitted for evaluation of abnormal LFTs to our unit (2001-2004). Clinical features, biochemical data, HCV and HBV status, histological features and iron status parameters were registered at baseline. All patients were genetically tested for *HFE* gene mutations. IR was determined by the homeostasis model assessment (HOMA) method using the following equation: insulin resistance (HOMA-IR) = fasting insulin (μU/mL) × fasting glucose (mmol/L)/22.5.

All patients had liver ultrasound (US); liver biopsy was performed only when clinically appropriate and in patients who did not refuse. Steatosis on US was assessed as present or absent; when present, it was graded as mild, moderate or severe by two experienced ultrasonographers (always the same throughout the study period), who were unaware of the clinical and laboratory results. The presence of steatosis was determined in a qualitative manner according to standardized criteria[25].

HFE mutation analysis

HFE gene mutations were evaluated by a reverse hybridization assay (Nuclear Laser Diagnostics) that assessed 11 *HFE* gene mutations: V53M, V59M, H63D, H63H, SC65C, C282Y, Q127H, E168Q, E168X, W169X, Q283P on DNA from peripheral blood mononuclear cells. Extracted DNA fragments were amplified by PCR and PCR products were hybridized with allele-specific oligonucleotide probes, and the hybridized probes were read by a colorimetric reaction.

Histological examination

Biopsies were evaluated for grade and stage according to Ishak[26] and, on Perl's Prussian-blue-stained sections, for iron content. Stainable iron was scored as: grade 0, no detectable iron; grade 1, granules of iron visible at 400 × magnification; grade 2, discrete iron granules visible at 100 × magnification; grade 3, iron visible at 25 × magnification, and grade 4, masses of iron visible at 10 × magnification.

Statistics analysis

Continuous variables were summarized as mean ± SD and categorical variables as frequency and percentage. Multiple logistic and linear regression models were used to assess the relationship of steatosis, high ferritin

Table 1 Demographic, laboratory and histological features of 124 patients (mean ± SD)

Variable	
Mean age (yr)	53.3 ± 1.2
Age (yr), *n* (%)	
≤ 50	51 (41.2)
> 50	73 (58.8)
Sex, *n* (%)	
Male	90 (72.5)
Female	34 (27.5)
BMI (kg/m^2)	
< 25	74 (59.6)
25-29.9	50 (40.3)
ALT-UNL	3.0 ± 1.0
AST-UNL	2.0 ± 1.0
GGT-UNL	2.0 ± 0.3
Ferritin (ng/mL)	799.7 ± 75.6
Serum iron (μg/dL)	126 ± 6.3
Platelet count × 10^3/cmm	186 ± 74.33
HOMA score	3.48 ± 1.80
Steatosis	92 (74.2)
Etiology	
Anti-HCV	53 (42.7)
NAFLD	35 (28.2)
NAFLD/diabetes	11 (8.8)
NASH	11 (8.8)
Cryptogenic	14 (11.3)
Histology (54)	
Chronic hepatitis C	27 (50)
Cirrhosis cryptogenic	9 (16.6)
NAFLD	7 (12.9)
NASH	11 (20.3)
HFE mutations	53 (42.7)
H63D heterozygous	49 (39.5)
C282Y heterozygous	2 (1.6)
C282Y/H63D compound het	2 (1.6)

ULN: Upper limit of normal.

and chronic liver disease. The dependent variable was steatosis on US, coded as 0 (absent) or 1 (present). As candidate risk factors for steatosis, we selected age, sex, BMI, presence of cirrhosis, baseline alanine aminotransferase (ALT)/aspartate aminotransferase (AST), platelets, GGT, ferritin, serum iron, transferrin, transferrin saturation, glucose, bilirubin, and diabetes. Multiple logistic regression analysis was performed to identify independent predictors of steatosis. Multiple linear regression analysis was performed to identify independent predictors of ferritin levels as a continuous dependent variable. Variables found to be associated with the dependent variables on univariate logistic or linear regression at $P \leq 0.10$ were included in multivariate regression models. Regression analyses were performed using PROC LOGISTIC and PROC REG subroutines (SAS Institute, Inc., Cary, NC, USA)[23].

RESULTS

Features of the patients included in the study are shown in Table 1. The 124 patients (34 women and 90 men) had a mean age of 53.3 ± 1.2 years. The mean value of ferritin was 799 ± 75 ng/mL and that of serum iron was 126 ± 6.3 μg/dL.

Table 2 Univariate analysis of risk factors for absent/present liver steatosis in 124 patients with high serum level ferritin

Variable	Steatosis		*P* value
	Absent (*n* = 32)	Present (*n* = 92)	
Age (yr)	50.9 ± 3.1	54.2 ± 1.3	0.06
Sex	18 (56.2)	72 (78.2)	0.14
BMI (kg/m^2)	24.4 ± 3.2	25.2 ± 3.1	0.30
ALT-UNL	47.7 ± 7.7	117.5 ± 11.2	0.1
AST-UNL	35.7 ± 5.3	89 ± 10.8	0.3
GGT	93.1 ± 20.7	174.1 ± 19.7	0.03
Anti-HCV positivity	24 (75)	28 (30.4)	0.02
Ferritin (ng/mL)	464 ± 183	1060.8 ± 79	0.0006
Serum Iron (μg/dL)	96.3 ± 7.5	137 ± 7.7	0.8
Platelet count × 10^3/cmm	217.8 ± 16.1	176.9 ± 8.26	0.24
HOMA	3.0 ± 2.25	3.5 ± 2.8	0.23
HFE mutations	14 (43.7)	40 (43.4)	0.63
Diabetes	11 (34.3)	11 (12)	0.2

HCV infection was detected in 53 patients (42.7%), 35 of whom (28.2%) had NAFLD without overt diabetes, 11 (8.8%) had NAFLD associated with diabetes, and 11 had NASH at histology. Finally, 14 patients (11.3%) were classified as having cryptogenic chronic hepatitis.

Overall, 92 patients (74.2%) had steatosis on US: 46 moderate and 46 severe. The etiological pattern of the patients with steatosis was as follows: 35 (38%) subjects were infected with HCV, 35 (38%) had NAFLD, 11 (12%) were diabetic with NAFLD, and 11 (12%) had a diagnosis of NASH at histology.

HCV infection was detected in 53 patients (42.7%). All these were infected by HCV genotype 1b; 36 (68%) had steatosis, nine were detected by US and 27 by liver biopsy.

At liver biopsy, performed in 54 patients out of 124 (43.5%), 27 (50%) had chronic hepatitis C and nine (16.6%) had micronodular cryptogenic cirrhosis. Seven patients (12.9%) had NAFLD (macrovesicular steatosis) and 11 (20.3%), NASH (macrovesicular steatosis and lobular inflammation). Seventeen patients (31.5%) had siderosis: eight, grade 1 and nine, grade 2.

Among the 11 *HFE* gene mutations analyzed, only two (H63D and C282Y) were present in our population, while the remaining nine mutations were not found in any patient. H63D and C282Y mutations were distributed as follows: 53 patients tested (42.7%) carried at least one *HFE* gene mutation. These were distributed as follows: 49 (39.5%) patients were H63D heterozygous, two (1.6%) were C282Y heterozygous, and two (1.6%) were C282Y/H63D, compound heterozygous. None were ultimately diagnosed with HH on clinical and laboratory criteria.

Univariate and multivariate analyses were performed to identify predictors of steatosis. By univariate analysis age ($P = 0.06$), ferritin ($P = 0.0006$), GGT ($P = 0.03$) and anti-HCV positivity ($P = 0.02$) were associated with steatosis ($P < 0.10$) (Table 2). By multivariate analysis, ferritin (OR: 1.002; 95% CI: 1.001-1.004), and GGT (OR: 1.007; 95% CI: 1.001-1.013) were the only independent predictors of steatosis (Table 3). The baseline ferritin

Table 3 Predictors of steatosis in 124 patients by logistic regression model

Predictor	Univariate analysis		Multivariate analysis	
	OR (95% CI)	*P* value	OR (95% CI)	*P* value
Age (yr)	0.962 (0.923-1.002)	0.060	0.97 (0.94-1.14)	0.23
Sex	0.455 (0.160-1.297)	0.14	-	-
BMI (kg/m^2)	1.050 (0.96-1.14)	0.23	-	-
ALT-UNL	0.997 (0.993-1.001)	0.12	-	-
AST-UNL	0.998 (0.994-1.002)	0.36	-	-
GGT-UNL	1.007 (1.001-1.013)	0.030	1.007 (1.003-1.014)	0.0043
Anti-HCV positivity	0.274 (0.110-0.682)	0.005	0.40 (0.20-1.10)	0.08
Platelet count × 10^3/cmm	1.000 (1.000-1.000)	0.24	-	-
Ferritin (ng/mL)	1.002 (1.001-1.004)	0.0006	1.003 (1.002-1.004)	0.0009
Serum Iron (μg/dL)	0.999 (0.991-1.008)	0.84	-	-
HFE mutations	0.796 (0.313-2.020)	0.63	-	-
Diabetes	0.995 (0.986-1.004)	0.28	-	-

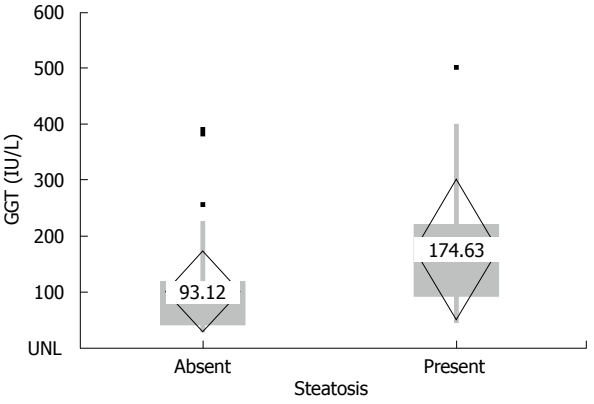

Figure 1 Baseline GGT levels according to steatosis in 124 non-obese, non-alcoholic patients without hereditary haemochromatosis (HH).

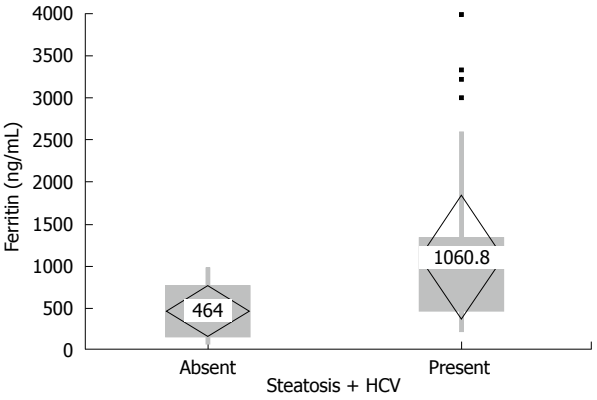

Figure 2 Baseline ferritin levels according to steatosis and HCV infection status in 124 non-obese, non-alcoholic patients without hereditary haemochromatosis (HH).

and GGT levels in patients with or without steatosis are shown in Figures 1 and 2.

To identify predictors of ferritin levels, univariate and multivariate linear regression analyses were performed. Univariate analysis showed that male sex, anti-HCV positivity, platelet count, AST, ALT, GGT level and steatosis were significantly associated with ferritin levels. The model for the independent predictors of ferritin levels as a continuous variable by multiple linear regression analysis (Table 4) included anti-HCV positivity (P = 0.0028), platelet count (P = 0.0161) and steatosis (P < 0.0001). Figure 2 outlines baseline ferritin levels according to HCV infection status.

Table 4 Multivariate analysis of risk factors for high serum ferritin levels in 124 patients by linear regression model

Variable	β	SE	*P*
Male	93.183	183.13	0.612
ALT-UNL	-0.07174	1.09734	0.948
AST-UNL	1.06027	1.05841	0.319
GGT-UNL	0.92457	0.5746	0.111
Anti-HCV positivity	521.964	169.40	0.0028
Platelet count × 10^3/cmm	-0.00250	0.0010	0.0161
Steatosis	933.7287	180.437	< 0.0001

DISCUSSION

Hyperferritinemia is frequent in patients with chronic liver disease, whatever the etiology of the underlying damage. In this cohort of 124 non-obese, non-alcoholic patients with high serum ferritin levels, without HH, the cause of liver disease was chronic HCV infection in 42.7%, NAFLD/NASH in 45.9%, and untraceable in 11%. Steatosis on US was predicted independently by ferritin and GGT levels. High ferritin levels were associated with HCV infection and with more advanced liver disease, shown by low platelet counts.

In our study, no patients could be finally diagnosed with HH, although at least one of the characterized *HFE* gene mutations (C282Y and/or H63D, mostly the latter) was found in 50% of our patients in an heterozygote state. In fact, none of these carriers of *HFE* mutations had a transferrin saturation > 45%, liver siderosis beyond grade 1, or evidence of any other organ damage attributable to iron overload. It is noteworthy that an excess H63D allele frequency observed in our patients, as compared to the 12%-19% range observed in the normal population in our area[5,28], suggests that heterozygosity for this mutated allele may increase the appearance of high ferritin levels, once predisposing factors such as IR, steatosis and cirrhosis are operating.

Chronic hepatitis C, with or without cirrhosis, often presents with abnormal iron indices[29,30], particularly

with raised levels of ferritin, which does not necessarily represents iron overload. Several mechanisms have been hypothesized to explain the altered iron indices and possible liver siderosis, including an excess of oxygen free radicals, increased fibrogenesis through activation of stellate cells and impairment of the host immune response[31-34]. Among our 29 patients with chronic liver disease caused by HCV genotype 1b, in whom liver biopsy was performed, only 17 had siderosis (eight mild, nine moderate, none severe). Theoretically, serum ferritin could be elevated as an acute phase reaction linked to the necroinflammatory process of chronic hepatitis C, but the moderate increase in ALT and the degree of activity typically observed in these patients negates this interpretation, even if in our analysis chronic HCV infection was independently linked to higher ferritin levels at multivariate analysis. It is however difficult to disentangle the role of HCV from that of steatosis, which is commonly associated with raised levels of ferritin[35,36], and is a common finding in HCV infection[37], even when caused by HCV genotype 1[14]. In our study, HCV-infected patients also showed a moderate degree of steatosis. NAFLD is known to be by itself strongly associated with the metabolic syndrome, which may explain the strong relationship between HCV infection and diabetes. The association between IR and moderate/severe steatosis in chronic hepatitis C is well supported[36-38]. In fact, IR could lead to the development of steatosis of the liver in HCV-infected patients[14], which makes them prone to the onset of diabetes.

In NAFLD, lipid peroxidation promotes transition from steatosis to steatohepatitis, which involves multiple cellular adaptations and evokes biomarkers of the oxidative stress that occurs when fatty acid metabolism is altered. The induction of heme-oxygenase 1 is an adaptive response against oxidative damage elicited by lipid peroxidation, and may be critical in the progression of the disease[39]. The association we found between ferritin and moderate/severe steatosis supports the concept that serum ferritin is a risk factor for fatty liver. Further support for this hypothesis is lent by the data of Zelber-Sagi *et al*[40] who demonstrated that NAFLD is the major determinant of increased serum ferritin levels at a population-based level. Moreover, they have shown that the association between serum ferritin and insulin is much more evident in the NAFLD group. Although recent studies have suggested that serum ferritin is a marker of IR[42-44], we could not provide evidence for a direct correlation between IR and elevated levels of serum ferritin. Consonant with Zelber-Sagi *et al*[40], we believe that the association found in previous studies between ferritin and IR may depend upon undiagnosed NAFLD.

Data from the third National Health and Nutrition Examination Survey (1988-1994) show a significant association between elevated serum ferritin and newly diagnosed diabetes mellitus[16]. We found that 17.7% of our patients had type 2 diabetes. In our study, however, ferritin levels were not significantly associated with IR, as evaluated by HOMA score, as well as by the presence of overt diabetes, probably as a result of the relatively small size of this sample, in which younger patients under evaluation for chronic hepatitis C predominated. Although a recent study has suggested that diabetes is the main factor accounting for the high ferritin level detected in chronic HCV infection[45], we could not provide evidence for a direct correlation between IR and hyperferritinemia.

An important finding of this work is the association we found between raised ferritin and reduction in platelet counts, a known marker of portal hypertension[46]. We confirmed the observation by Bugianesi *et al*[36] who demonstrated that serum ferritin, but not iron stores, was a significant predictor of severe fibrosis in patients with NAFLD. All these data provide further evidence that hyperferritinemia might be another surrogate marker of advanced liver disease of any etiology.

According to recent reports, GGT is an independent predictor of liver steatosis[14]. Our data indicate that patients with elevated GGT levels have the greatest likelihood of having moderate/severe steatosis. The administration of a questionnaire regarding alcohol intake and drug use or abuse makes us confident in excluding any role of these potential confounders on GGT levels. Lack of data on smoking, however, could affect the accuracy of the results[47]. The association between GGT levels and steatosis is likely the result of the association between regional body fat distribution and fatty liver, irrespective of total body fat quantity, which is consistent with the assumption that GGT is a surrogate marker of central fat accumulation. Therefore, the GGT level may be a simple and reliable marker of visceral and hepatic fat and, by inference, of hepatic IR. Thus, patients with elevated serum ferritin and GGT levels are at risk of developing liver steatosis[48]. Modelling the indication for US scanning on these predictors would maximize its cost effectiveness.

The main limitation of the current study, as well as of other cross-sectional studies, is that it is unable to distinguish the temporality of the associations between hyperferritinemia, steatosis and chronic hepatitis C. Lack of histological data in a proportion of subjects, particularly on intra-hepatic iron deposition, could also affect the interpretation of our findings. We are aware that the use of a more sensitive imaging technique such as magnetic resonance imaging could improve the rate of steatosis detection. In addition, we cannot exclude the possibility that denied alcohol abuse may be responsible for the observed prevalence of steatosis. A further methodological issue arises in the potential limitation of the generalizability of our results to new populations and settings. Our study included a Mediterranean cohort of non-obese patients without HH, which limits the broad application of the results.

In conclusion, this study shows that in a non-obese cohort of non alcoholic patients, steatosis and chronic HCV infection are the main causes of hyperferritinemia. In Southern European populations, the finding of high

ferritin levels, after the exclusion of diagnosis of HH, represents a risk factor for steatosis and has clinical relevance, being associated with low platelet count.

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COMMENTS

Background

Patients with chronic hepatitis C virus (HCV) infection often have elevated serum iron indices, but these do not accurately reflect hepatic iron content, nor are they able to predict clinically important endpoints, such as progression of fibrosis and responsiveness to interferon-based regimens.

Research frontiers

In this study, the authors showed that, in a non-obese cohort of non-alcoholic patients, steatosis and chronic HCV infection are the main causes of hyperferritinemia. In southern European populations, high ferritin levels, after exclusion of a diagnosis of hereditary hemchromatosis (HH), represent a risk factor for steatosis and have clinical relevance, being associated with low platelet count.

Innovations and breakthroughs

In a non-obese cohort of non-alcoholic patients with chronically abnormal liver function tests, without HH, serum ferritin high level is, therefore, a risk factor for steatosis.

Applications

Hyperferritinemia can be used as markers of steatosis in non-obese and non-alcoholic patients.

Peer review

The authors study the underlying liver disease in a cohort of individuals selected because they had both chronic liver disease as well as elevated serum ferritin levels. They found that most individuals had either chronic HCV or fatty liver disease. Additional analysis of clinicopathological data showed an association between ferritin and steatosis and GGT and steatosis. Overall the paper is well written.

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Evaluation of a rabbit rectal VX2 carcinoma model using computed tomography and magnetic resonance imaging

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Abstract

AIM: To establish a rabbit rectal VX2 carcinoma model for the study of rectal carcinoma.

METHODS: A suspension of VX2 cells was injected into the rectum wall under the guidance of X-ray fluoroscopy. Computed tomography (CT) and magnetic resonance imaging (MRI) were used to observe tumor growth and metastasis at different phases. Pathological changes and spontaneous survival time of the rabbits were recorded.

RESULTS: Two weeks after VX2 cell implantation, the tumor diameter ranged 4.1-5.8 mm and the success implantation rate was 81.8%. CT scanning showed low-density foci of the tumor in the rectum wall, while enhanced CT scanning demonstrated asymmetrical intensification in tumor foci. MRI scanning showed a low signal of the tumor on T_1-weighted imaging and a high signal of the tumor on T_2-weighted imaging. Both types of signals were intensified with enhanced MRI. Metastases to the liver and lung could be observed 6 wk after VX2 cell implantation, and a large area of necrosis appeared in the primary tumor. The spontaneous survival time of rabbits with cachexia and multiple organ failure was about 7 wk after VX2 cell implantation.

CONCLUSION: The rabbit rectal VX2 carcinoma model we established has a high stability, and can be used in the study of rectal carcinoma.

Key words: Rectal carcinoma; Animal model; Rabbit; VX2; Computed tomography; Magnetic resonance imaging

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INTRODUCTION

Rectal carcinoma is a common malignant tumor of the gastrointestinal tract. Imaging examination plays an important role in its identification, diagnosis, preoperative staging, treatment decision, and postoperative assessment[1-3]. Currently, experimental animal models of rectal carcinoma are often induced by chemical carcinogens[4-6]. This kind of methods requires lots of time and individual variations can be very large. In this study, a rabbit rectal carcinoma model was established and evaluated, which can be monitored dynamically by computed tomography (CT) and magnetic resonance imaging (MRI) and used in diagnosing and staging rectal carcinoma.

MATERIALS AND METHODS

Experimental animals

Twenty-two (4-5 mo old) New Zealand white rabbits, weighing 2.4-2.9 kg, were used in this study, and the breeding rabbits were donated by Professor Bin Hu,

Department of Ultrasound, Sixth People's Hospital of Shanghai Jiaotong University, China.

Preparation for surgery

Experimental rabbits were lavaged 24 h prior to surgery. Mannitol (20%) was prepared with warm water at a ratio of 1:1 and the lavage dose was approximately 250 mL per rabbit. Lavaged rabbits were fasted with free access to water prior to surgery. VX2 tumor cells were grown in the hind leg muscle of rabbits and harvested for the preparation of suspended tumor cells at a concentration of 1-2 × 10^7/mL.

Establishment of rectal carcinoma model

Experimental rabbits were anesthetized with 30 mg/kg pentobarbital sodium *via* the ear vein. Rabbits were placed at a dorsal position with their legs fixed. A 7-cm long sterilized plastic hollow pipe, 7 mm in diameter, was inserted into the anus to brace the rectal cavity. A 22G transfixion pin was injected into approximately 4-5 cm of the rectal wall around the anus. A contrast medium (0.2 mL, Ultravist 300) was injected with its distribution monitored by X-ray fluoroscopy. If its border was ill-defined and dispersed, the needle point would be in a gap region between the outside of the organ and the rectal wall. Then, the puncture needle was reinserted into the rectal wall until the border of contrast medium became sharply margined. At this point, 0.2 mL of suspended VX2 cells was injected, then 0.1-0.2 mL of normal sodium was injected to fully rinse all the VX2 cells into the rectal wall. After 5 min, the needle was withdrawn slowly. The rabbits were allowed to have normal food following recovery from anesthesia.

CT and MRI scanning of tissue sections

Rabbits were anesthetized with 30 mg/kg pentobarbital sodium before CT and MRI scanning of tissue sections at 2-, 3-, 4-, 5- and 6-wk intervals after VX2 cell implantation. CT scanning was performed using a GE LIGHT SPEED VCT 64 CT set with the following parameters: 80 kV, 100 mA, 14-16 cm in field of view (FOV), 512*512 matrix, 1.25 mm section thickness, and 1.25 mm section interval. A contrast medium (Ultravist 300) was injected at 0.5 mL/s and 1.5-2.0 mL/kg. Arterial phase scanning was started 15 s after contrast medium injection and after 30 s during the portal venous phase. The image was processed at the ADW4.0 workstation. MRI scanning was performed by a Philips Achieva 3.0 imager, with the rabbit placed at a supine position in a phased-array articular genu coil. MRI sequences included the pre-contrast T_1W-TSE, gadolinium-enhanced T_1W-TSE, and T_2W-TSE sequences in the axial plane (TR-2727 ms, TE-100 ms, 2.0 mm section thickness 2.0 mm, and section interval 0.8 mm), T_2_TSE_SPAIR sequence in the axial plane (TR-4341 ms, TE-62 ms, section thickness 2.0 mm, and section interval 0.2 mm), and PD_SPAIR sequence in the coronal planes (TR-4710 ms, TE-30 ms, section thickness 2.0 mm, and 0.2 mm section interval 0.2 mm). The contrast medium (Magnevist) was injected at 0.5 mL/s and 1.5-2.0 mL/kg. Enhancement scanning was started 20 s after contrast medium injection, and the image was processed at a View Forum R5.1 V1L1 workstation.

Measurement of tumor volume

Gross tumor volume (V) was measured following the equation: $V = 0.5 (a \times b^2)$, where a represents the maximum tumor diameter, and b represents the minimum tumor diameter. Tumor growth rate (TGR) was calculated following the equation: $TGR = (V_2 - V_1)/V_1 \times 100\%$, where V_1 represents the gross tumor volume measured at an earlier time point and V_2 represents the gross tumor volume measured at a later time point.

Histopathological changes in rabbit rectal VX2 carcinoma model

Three rabbits were sacrificed after each CT and MRI scanning at 2-6 wk intervals after VX2 cell implantation for observation of pathological changes in the rectal VX2 carcinoma model. Autopsies were also performed after spontaneous death of the rabbits. Tumor location, size, activity, circumscription, and metastasis were observed grossly. The rectum-implanted tumor and the major organs involved were fixed in formalin and embedded in paraffin. Tumor tissue was cut into sections, which were stained with hematoxylin-eosin (H&E), and evaluated under a light microscope.

Statistical analysis

Data were presented as mean ± SD. Gross tumor volumes at an earlier and later time point were compared by Student's *t* test. Statistical analyses were performed using SPSS 11.0 software. $P < 0.05$ was considered statistically significant.

RESULTS

Twenty-two New Zealand white rabbits were used to establish the model. Eighteen of them developed primary tumors with a success rate of 81.8%.

CT detection

Tumor implanted in the rectal wall of each rabbit could be detected by CT scanning 2 wk after VX2 cell implantation. The appearance of rectal enteric cavity at this time point was still normal without obvious stricture. However, part of the rectal wall exhibited irregularly intensified armillary after enhancement (Figure 1A). The gross tumor volume was increased 3 wk after VX2 cell implantation, and appeared as a small lump with low density or isodensity on CT images. The boundary between the tumor and normal rectal wall could not be clearly distinguished. However, the rectal enteric cavity became elliptical with stricture, allowing the tumor margin to be distinguished from its surrounding tissue (Figure 1B). After 4 wk, the gross tumor volume was increased, the rectal wall was thickened, and the rectal enteric cavity became flatter, with increased stricture.

Table 1 Gross tumor volume and TGR after tumor implantation

Time after implantation	a (mm)	b (mm)	V (mm^3)	TGR (%)
2 wk	5.029 ± 0.544	4.129 ± 0.475	46.180 ± 14.583	-
3 wk	16.783 ± 1.387	9.942 ± 1.326	848.239 ± 270.715	1736.8
4 wk	19.419 ± 1.150	15.800 ± 1.255	2443.569 ± 480.966	185.7
5 wk	24.763 ± 1.762	22.163 ± 1.388	6163.157 ± 1181.274	159.3

Tumor volume (V) = 0.5 ($a \times b^2$), where a and b represent the maximum and minimum tumor diameters, respectively; TGR = (V_2 - V_1)/V_1 × 100%, where V_1 represents the gross tumor volume measured at an earlier time point and V_2 represents the gross tumor volume measured at a later time point.

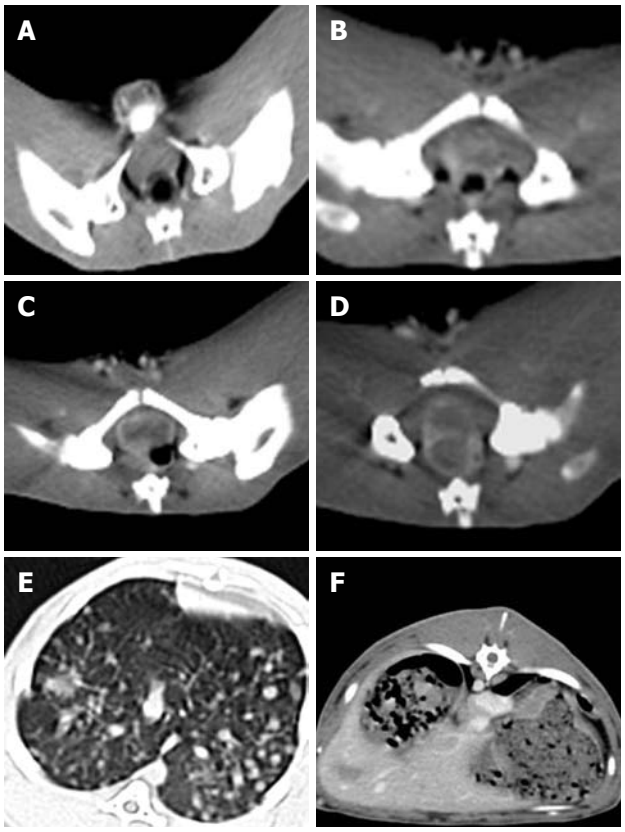

Figure 1 CT enhancement scanning images of rectal wall 2(A), 3(B), 4(C), and 5(D) wk after VX2 cell implantation in the experimental rabbits, and images of metastatic nodes detected in the lung (E) and liver (F), respectively.

Necrosis could be detected in the middle of the tumor, and the surrounding tissue was involved at different degrees. CT scanning showed that the tumor appeared to have an intensified, solid marginal zone and a central region with low density but without intensification. In contrast, the surrounding tissue was intensified as the tumor (Figure 1C). CT scanning revealed significant stricture of the tumor, which was fixed to the pelvic wall and rectal enteric cavity 5 wk after VX2 cell implantation (Figure 1D). After 6 wk, the rectal enteric cavity was almost compressed to the point of closure and metastatic nodes were detected in the lung (Figure 1E) and liver (Figure 1F), as in the seroperitoneum. The metastatic nodes appeared in the lung earlier and much more obviously than in the liver, since the blood supply in the lower part of rectum returns to the inferior vena cava but not to the hepatic portal vein.

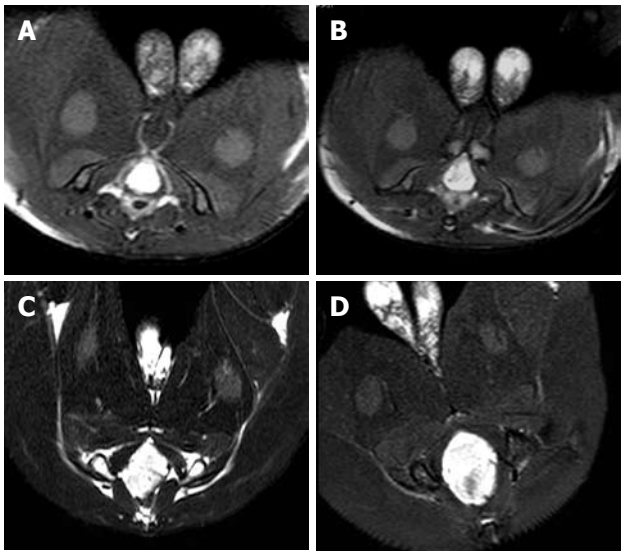

Figure 2 MRI of T2-TSE-SPAIR 2(A), 3(B), 4(C), and 5(D) wk after VX2 cell implantation in the experimental rabbits.

MRI detection

MRI showed that the signal of VX2 tumor was low on T_1-weighted imaging (T_1WI), resulting in the detection of an indistinct boundary, and high on T_2-weighted imaging (T_2WI), allowing clear visualization of the boundary. In addition, the signal of VX2 tumor in PD sequence was higher than that on T_1WI. Tumor boundary could be distinguished from its surrounding tissue after enhancement. Necrosis with low signals, but without intensification after enhancement, could be detected in the middle of the primary tumor 4 wk after VX2 cell implantation. MRI and CT demonstrated similar growth and metastasis of the tumor. However, MRI identified more precisely the tumor boundary, size and infiltration, and infection foci than CT scanning. MRI of the tumor at 2-, 3-, 4-, and 5-wk intervals after VX2 cell implantation are shown in Figure 2. The gross tumor volume (V) and the TGR at these time points were also calculated (Table 1). The TGR at each time point was quite different ($P < 0.0001$), but the fastest growth of tumor was observed 3 wk after VX2 cell implantation.

Histopathological changes

Macroscopic image of the resected tumor appeared as a single node with an obscure boundary and affluent vascellum (Figure 3A and B). Metastasis

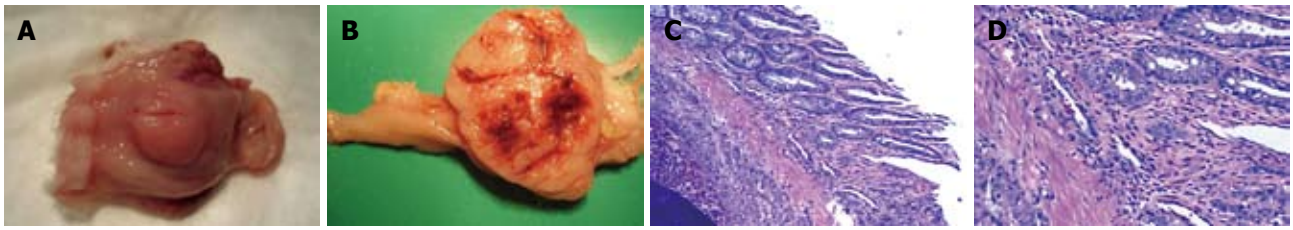

Figure 3 Characteristics of isolated rectal carcinoma specimens (A, B) and light microscope images of HE stained tissue sections (C, × 40; D, × 100).

outside the rectal wall was not detected until 4 wk after VX2 cell implantation. The rectal wall was thickened due to different degrees of enteric cavity stricture. No enterectasis or rectum obstruction was observed. Necrosis appeared in the middle of the tumor 4 wk after VX2 cell implantation, with enteric cavity stricture and enterectasis, as well as metastasis outside the rectal wall in the pelvic cavity. Metastases to the lung and liver, and seroperitoneum and rectum obstruction were detected 6 wk after VX2 cell implantation. However, the metastasis in the liver was not as obvious as that in the lung, and sometimes it was not detectable, because the metastasis in the liver was not sufficiently large to be visualized. The experimental rabbits developed cachexia and multiple organ failure, and died spontaneously about 7 wk after VX2 cell implantation.

Tumor tissue sections were stained with H&E and evaluated under a light microscope. Infiltrating tumor cells were visible and the interstitial tissue boundary was not distinct. Minimal connective tissue was observed, and dispersed tumor cells were found with separation of fibers. Fresh capillaries were abundant (Figure 3C), consistent with the large, irregular tumor volume. Cytoplasm of the tumor cells was abundant, and pale red in color. There was an abnormal number of mitotic nuclei. Hypertrophic nuclei were also found, varying in shape, size, and color (Figure 3D). Two weeks after VX2 cell implantation, the tumor grew in the rectal wall with no involvement of its peripheral tissue. However, by 3 wk after VX2 cell implantation, the tumor grew through the rectal wall with the mesorectal fascia tissue involved 4 wk after VX2 cell implantation.

DISCUSSION

Since lymph in the gastrointestinal tract is very rich, the survival rate of heterogeneic tumor tissue transplanted to the intestine wall of experimental animals is practically zero. Because of this, smaller animal models are often used in the study of rectal carcinoma. Experimental animal models can be established by repeated injection of chemical carcinogens into the abdominal cavity of animals, or repeated lavaging of the intestinal tract. However, these methods are time consuming and their success rate is low. Furthermore, these small animal models cannot contribute to the diagnosis of rectal carcinoma.

VX2 cells can be implanted into rabbits where they can grow. It has been shown that this cell line, implanted into the muscle, kidney, liver, lung, pleura, ossature, and mammary gland of rabbits, can produce an *in situ* tumor model that mimics the human condition[7-12]. The implantation techniques for VX2 cells include implanting a small lump of VX2 tumor tissue and injecting a suspension of VX2 cells directly or under the guidance of B-mode ultrasound or CT. Wang *et al*[13] demonstrated that laparotomy could be used to establish a rabbit model of rectal VX2 carcinoma, with a success rate of 60%. Considering the substantial damage caused by laparotomy, we established the rabbit model of rectal VX2 carcinoma by injecting a suspension of VX2 cells into the rectal wall through the skin of the anorectum under the guidance of X-ray fluoroscopy. CT or MRI showed the implanted tumor in the rectal wall 2 wk after VX2 cell implantation. The involved tissue around the rectum was observed and metastases to the lung and liver were detectable 4 and 6 wk, respectively, after VX2 cell implantation. The success rate of this method was 81.8%.

This rabbit rectal VX2 carcinoma model was evaluated by CT scanning, MRI, and histopathology.

CT has many advantages in monitoring rectal tumor by displaying its location, size, shape, peripheral tissue and lymph node involvement[14,15]. Recently, with the update of CT instruments and CT imaging techniques, the sensitivity and specificity of CT in detection of tumors have been greatly improved. Multi-section CT (MSCT) is more advantageous than ordinary CT, by reducing the shadow of motion and displaying dynamic enhancement effects[16-18]. Furthermore, CT plays an important role in preoperative staging of rectal carcinoma, especially in detecting metastasis in the lung and liver[19]. CT scanning has been recommended to patients with colorectal cancer[20-22]. In this study, MSCT showed the growth of tumor and its surrounding tissue, as well as distant organ metastasis, suggesting that CT scanning is an ideal method for monitoring VX2 rectal carcinoma.

Since the location of the rectum is relatively fixed, tumor tissue can be observed by contrast with the peripheral fat, and is seldom affected by the shadows that result from respiration. MRI is a good imaging technique for detection of rectal tumor and can show the layers of the rectal wall, including the mucosa with a low-intensity signal, submucosa with a high-intensity signal, muscularis propria with a weak-intensity signal, perirectal fat with a high-intensity signal, and mesorectal fascia with a low-intensity signal. T_1WI can be used to evaluate fatty infiltration around the rectum, while T_2WI can display the infiltration depth in the rectal wall

and the relation between inherent muscle layers and mesorectal fascia. The most significant advantage of MRI in rectal carcinoma staging is its ability to describe the correlation between tumor and mesorectal fascia[23-25]. MRI can determine the circumferential resection margin (CRM)[26]. Induction of 3.0T magnetic resonance and improvement in phased-array coils make MRI display the CRM much more precisely[27,28]. Its accuracy for the prediction of CRM is consistent with histopathological assessment of specimens after surgery[29-32]. It has also been reported that MRI can predict the infiltration depth of rectal tumor in the range of 0.5 mm[33], which is consistent with histopathology results. MRI is more sensitive in detecting early stage tumor growth than CT, especially in measuring the tumor size. In addition, MRI can display metastasis of tumor to lymph nodes.

This animal model is easy to establish, reproducible, and induces minimal damage to experimental animals. In addition, the tumor growth time is short. The growth and metastasis of rectal VX2 carcinoma in rabbits are similar to those in humans. Therefore, it can be used in the study of rectal carcinoma.

COMMENTS

Background

Currently, experimental animal models of rectal carcinoma are often induced by chemical carcinogens, which is time consuming. It has been shown that implantation of VX2 cells into the muscle, kidney, liver, lung, pleural, ossature, and mammary gland of rabbits can produce an *in situ* tumor model that mimics the human condition.

Research frontiers

The implantation techniques for VX2 cells include implanting a small lump of VX2 tumor tissue and injecting a suspension of VX2 cells directly, or under the guidance of B-mode ultrasound and computed tomography.

Innovations and breakthroughs

It is feasible to establish a rabbit rectal VX2 carcinoma model by injecting a suspension of VX2 cells into the rectum wall under the guidance of X-ray fluoroscopy. This model is similar to human rectal carcinoma models in terms of tumor pathology, development, and metastasis.

Applications

This rabbit rectal VX2 carcinoma model can be used in examination, staging and diagnosis of rectal carcinoma.

Terminology

VX2 cell strain, a squamous carcinoma strain induced by Shope virus, can be implanted in rabbits.

Peer review

The animal model presents many analogies to human rectal carcinoma in terms of pathological findings and tumor development.

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Clinicopathological significance of B-cell-specific Moloney murine leukemia virus insertion site 1 expression in gastric carcinoma and its precancerous lesion

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Abstract

AIM: To explore the relation between B-cell-specific Moloney murine leukemia virus insertion site 1 (Bmi-1) expression and the clinicopathological features of gastric carcinoma (GC).

METHODS: Immunohistochemistry was used to detect the expression of Bmi-1 and ki-67. Double-labeling staining was used to display the distribution of Bcl-2$^+$/ki-67$^-$ cells in 162 cases of GC and its matched normal mucosa and precancerous lesion.

RESULTS: The positive rate of Bmi-1 expression in GC (52.5%) was significantly higher than that in normal gastric mucosa (21.6%, χ^2 = 33.088, P < 0.05). The Bmi-1 expression in GC was closely related with the Lauren's and Borrmann's classification and clinical stage (χ^2 = 4.400, 6.122 and 11.190, respectively, P < 0.05). The expression of ki-67 was related to the Borrmann's classification (χ^2 = 13.380, P < 0.05). Bcl-2 expression was correlated with the Lauren's classification (χ^2 = 4.725, P < 0.05), and the Bmi-1 expression both in GC (r_k = 0.157, P < 0.05) and in intestinal metaplasia (r_k = 0.270, P < 0.05).

CONCLUSION: Abnormal Bmi-1 expression in GC may be involved in cell proliferation, apoptosis and cancerization. This marker can objectively indicate the clinicopathological characteristics of GC.

Key words: B-cell-specific Moloney murine leukemia virus insertion site 1; Gastric carcinoma; Precancerous lesion; Cell proliferation; Apoptosis

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INTRODUCTION

B-cell-specific Moloney murine leukemia virus insertion site 1 (Bmi-1) is a transcriptional repressor belonging to the polycomb group gene family[1], which is a potent negative regulator of the Ink4a/Arf locus. Bmi-1 regulates cell proliferation and apoptosis and is over-expressed in several human tumors[2,3]. Reinisch *et al*[4] reported that Bmi-1 protein is expressed in stem cells, specialized cells and tumors of the gastrointestinal tract. In the present study, the expressions of Bmi-1, ki-67 and Bcl-2 were detected immunohistochemically. The distribution of Bcl-2$^+$/ki-67$^-$ cells was observed in gastric carcinoma (GC) and its matched normal mucosa as well as precancerous lesion. The relation between Bmi-1 expression and clinicopathological features of GC was explored.

MATERIALS AND METHODS

Clinicopathological data

Specimens were collected from 162 cases of GC with its matched normal gastric mucosa, 82 cases of intestinal metaplasia (IM), and 52 cases of dysplasia from the First Affiliated Hospital of China Medical University during August 2006-May 2008. The age of the patients was 30-80 years. According to the WHO's histological classification of gastric cancer, the 162 cases of GC were classified as four of papillary adenocarcinoma, 12 of well-differentiated adenocarcinoma, 50 of moderately differentiated adenocarcinoma, 75 of poorly differentiated adenocarcinoma, 10 of mucinous adenocarcinoma, nine of signet ring cell carcinomas and two of undifferentiated adenocarcinoma. Samples were fixed in 10% formalin, embedded in paraffin, cut into 4-μm thick sections and constructed in four blocks for tissue microarray. All the samples were evaluated by two experienced pathologists for diagnosis.

Immunohistochemistry

Expression of Bmi-1 and ki-67 in the specimens was detected using the PV-9000 kit (Beijing Zhongshan Goldenbridge Biotechnology Company) following its manufacturer's instructions. The working anti-human rabbit Bmi-1 polyclonal antibody (Abcam, USA) was diluted at 1:80. Anti-human mouse monoclonal antibodies ki-67 and Bcl-2 (ready to use) and double-labeling staining kit were purchased from Fuzhou Maixin Company (China). Immunohistochemical double-labeling staining was used to display the distribution of Bcl-2^+/ki-67^- cells. Antigens were retrieved after they were placed in a pressure cooker at a full pressure for 160 s in citrate buffer (pH 6.0). All procedures were implemented according to their manufacturer's instructions, respectively. For negative controls, sections were processed as above but treated with 0.01 mol/L phosphate-buffered saline instead of primary antibodies.

Two hundred cells from two selected representative fields of each section were counted by two independent observers for the determination of their immunostaining intensity. Staining intensity was classified as 0 (negative), 1 (weak), 2 (moderate), and 3 (strong). Half of the positive cells were quantified as a percentage of the total number of the same kind of cells counted in two high-power fields (× 400), and defined as 0: < 5%, 1: 5%-25%, 2: 26%-50%, 3: 51%-75% and 4: > 75%. Immunostaining intensity was divided into 0: negative (-), 1-4: weakly positive (+), 5-8: moderately positive (++), and 9-12: strongly positive (+++). A 5-bromo-4-chloro-3-indolyl-phosphate/nitro-blue tetrazolium (BCIP/NBT) and 3-amino-9-ethylcarbozole (AEC) double staining system was used to display Bcl-2^+/ki-67^- cells. Red fine granules in cytoplasm with unstained nuclei in the same cells were defined as Bcl-2^+/ki-67^- cells. Photoes were taken with a digital camera (Olympus AX70, Japan).

Table 1 Expression of Bmi-1 in normal gastric mucosa, GC and precancerous lesion

Disease features	Cases (*n*)	Positive Bmi-1 expression rate			χ^2	*P*
		-	+-+++	+-+++ (%)		
N	162	127	35	21.6	74.844[a]	< 0.001
IM	82	17	65	79.3	16.510[b]	< 0.001
DYS	52	10	42	80.8	59.819[c]	< 0.001
					13.067[b]	
GC	162	77	85	52.5	33.088[c]	< 0.001

[a]$P < 0.001$ *vs* IM; [b]$P < 0.001$ *vs* GC; [c]$P < 0.001$ *vs* N. N: Normal gastric mucosa; DYS: Dysplasia.

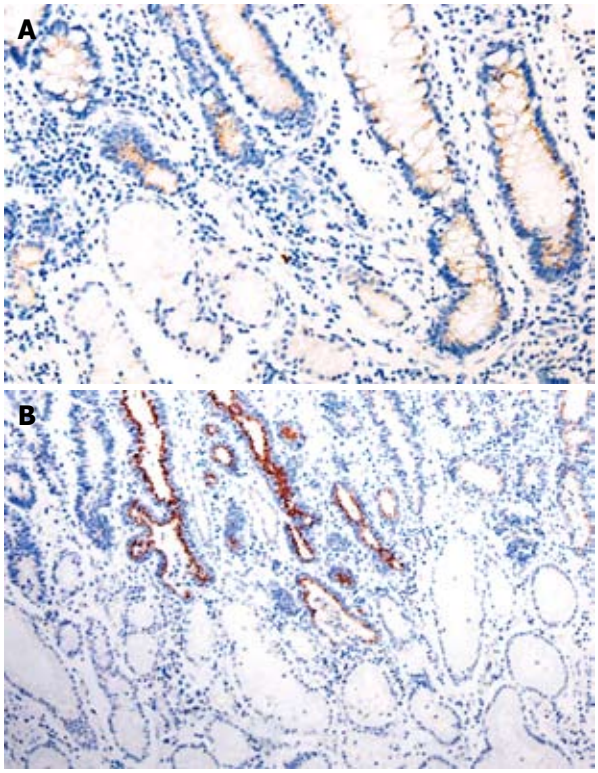

Figure 1 Expression of Bmi-1 in IM (A, × 200) and GC (B, × 100) (PV-9000).

Statistical analysis

Statistical analysis was performed using SPSS 11.5. χ^2 test was used to differentiate the rates of different groups and Kendall's tau-b rank correlation. $P < 0.05$ was considered statistically significant.

RESULTS

Bmi-1 expression in normal gastric mucosa, GC and precancerous lesion, and its relation with GC

The positive rate of Bmi-1 expression was 52.5%, 79.3%, and 80.8%, respectively, in GC, IM, and dysplasia (DYS), which was significantly higher than that (21.6%) in normal gastric mucosa (χ^2 = 33.088, 74.844, 59.819, respectively, $P < 0.05$). The immunoreactivity to Bmi-1 protein was located in the cytoplasm (Table 1, Figure 1).

The expression of Bmi-1 was related to the Lauren's and Borrmann's classification and clinical tumor stage

Table 2 Correlation between Bmi-1 expression and clinicopathological features of GC

Group	Cases (*n*)	Positive Bmi-1 expression rate			χ^2	*P*
		-	+-+++	+-+++(%)		
Gender					2.880	0.090
Male	116	60	56	50.0		
Female	46	17	29	65.2		
Age (yr)					3.658	0.056
≤ 60	82	46	36	43.9		
> 60	80	31	49	61.3		
Clinicopathological classification					11.190	0.001
EGC	30	6	24	80.0		
AGC	132	71	61	46.2		
Gross classification						
EGC						0.641
Ⅰ + Ⅱc	19	3	16	84.2		
Ⅲ	11	3	8	72.7		
AGC					6.122	0.013
Bor Ⅰ + Bor Ⅱ	25	19	6	24.0		
Bor Ⅲ + Bor Ⅳ	107	52	55	51.4		
WHO histological classification						< 0.001
PA	4	0	4	100.0		
WDA	12	3	9	75.0		
MDA	50	22	28	56.0		0.330[a]
PDA	75	42	33	44.0		0.063[b]
SRC	10	5	5	50.0		
MA	9	4	5	55.6		
UA	2	1	1	50.0		
Lauren's classification					4.400	0.036
Intestinal type	75	29	46	61.3		
Diffuse type	87	48	39	44.8		
Lymph node metastasis					3.042	0.081
No	98	52	46	46.9		
Yes	64	25	39	60.9		

Fisher's exact test, [a]*P* = 0.330 *vs* PDA; [b]*P* = 0.063 *vs* PDA. EGC: Early gastric carcinoma; AGC: Advanced gastric carcinoma; PA: Papillary adenoma; WDA: Well-differentiated adenoma; MDA: Moderately differentiated adenoma; PDA: Poorly differentiated adenoma; MA: Mucinous adenoma; UA: Undifferentiated adenoma.

(χ^2 = 4.400, 6.122, 11.190, $P < 0.05$), but not related to the age and gender of patients, and lymph node metastasis of GC (Table 2).

Expression of ki-67 and distribution of Bcl-2$^+$/ki-67 cells in normal gastric mucosa, GC and precancerous lesion

The immunoreactivity to Bcl-2 and ki-67 was located both in the cytoplasm (red fine granules) and in nuclei (dark blue fine granules), respectively. Most Bcl-2$^+$/ki-67$^-$ cells were distributed in the proliferating zone of gastric mucosa. The expression of ki-67 and Bcl-2 was correlated to the Borrmann's and Lauren's classification (χ^2 = 13.380 and 5.552, $P < 0.05$, Table 3).

Relation between expressions of Bmi-1, ki-67 and Bcl-2 in GC and IM

A positive relation was observed between Bmi-1 and Bcl-2 expressions in GC (r_s = 0.157, P = 0.043) and IM (r_s = 0.270, P = 0.038) (Figures 2 and 3, Table 4).

DISCUSSION

The Bmi-1 proto-oncogene is a transcriptional repressor, which can be discovered by retroviral insertion mutagenesis when transgenic mice are infected with Moloney murine leukemia virus[1]. It has been shown that Bmi-1 plays an important role in sustaining self-renewal of cell activity by repressing the *INK4A* locus that encodes $p16^{INK4A}$ and $p19^{ARF}$ in humans[5]. $P16^{INK4A}$ and $p19^{ARF}$ are capable of inducing growth arrest, cellular senescence and apoptosis. Several studies suggested that the pro-survival and pro-proliferation actions of Bmi-1 may be related to its ability to suppress the expression of proteins that regulate cell cycle progression. For example, in some cell types, when Bmi-1 is absent, the levels of $p16^{INK4A}$ and $p19^{ARF}$ increase[6]. Our study showed that the expression rate of Bmi-1 was 52.5%, 79.3%, and 80.8%, respectively, in GC, IM, and DYS, which was significantly higher than that (21.6%) in normal gastric mucosa ($P < 0.05$), indicating that Bmi-1 expression is involved in the mechanism that determines malignant potential[6], and may play a role in the occurrence and development of GC. In the absence of Bmi-1, $p16^{INK4A}$ may be up-regulated, leading to cell cycle arrest, senescence or apoptosis. In contrast, deregulation of *INK4a* allows cell cycle progression. $p19^{ARF}$ prevents the degradation of p53 by sequestering the p53-inhibitor MDM2, thereby allowing p53-mediated cell cycle arrest and apoptosis[7]. Since *INK4a-ARF* is the critical downstream target of Bmi-1 in the regulation of cell proliferation and apoptosis[7], and the stability of cells is

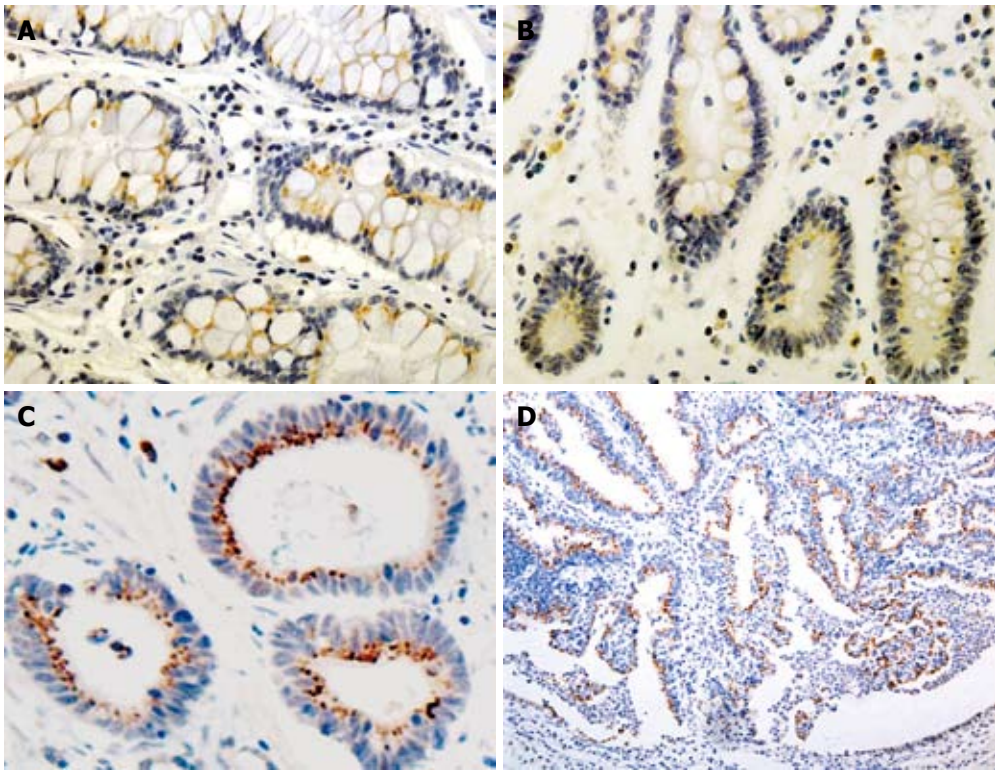

Figure 2 Expression of Bmi-1 in IM (A), mild DYS (B), gastric tubular adenocarcinoma (C) and papillary adenocarcinoma (D) (PV-9000 A-C × 400, D × 200).

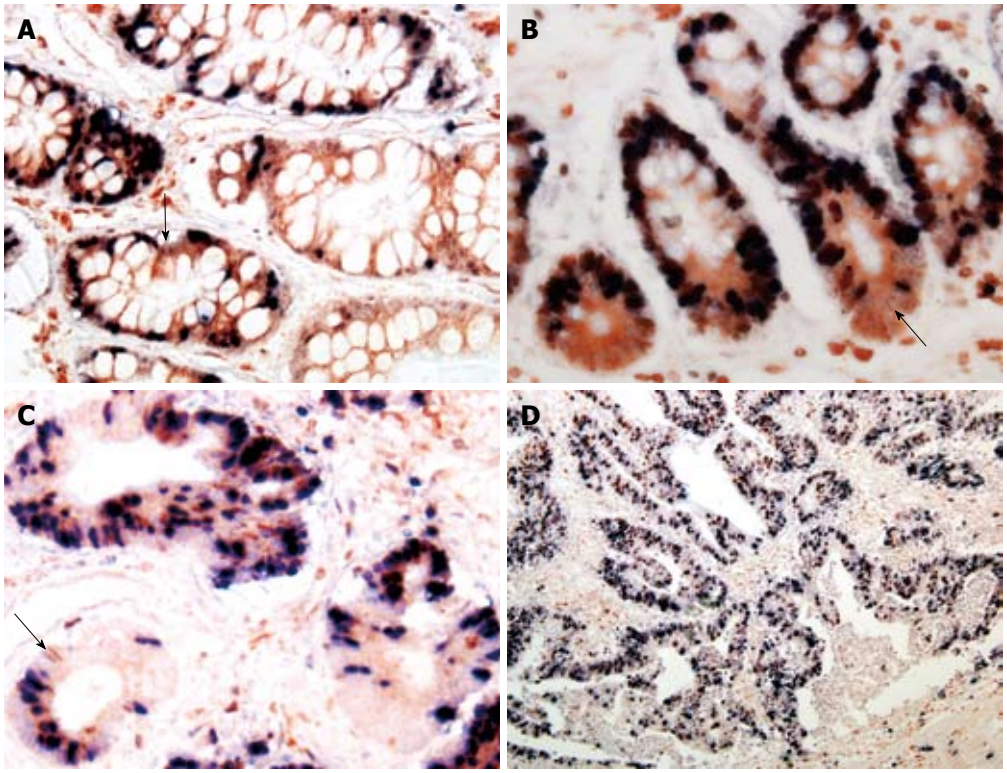

Figure 3 Distribution of Bcl-2$^+$/ki-67$^-$ cells in IM (A), mild DYS (B), gastric tubular adenocarcinoma (C) and papillary adenocarcinoma (D). Red fine granules in cytoplasm and unstained nuclei were defined as Bcl-2$^+$/ki-67$^-$ cells as shown by the arrows (Immunohistochemical double staining, A-C × 400, D × 200).

impaired, cancerization may be increased. In this study, Bmi-1 expression was significantly higher in gastric precancerous lesion than in normal gastric mucosa, indicating that Bmi-1 plays a role in the malignant transformation of gastric mucosa cells[6].

In the present study, over-expression of Bmi-1 occurred in high-grade intraepithelial dysplasia and colon cancerous cells, which is consistent with the reported findings[6]. This study also showed that the expression of Bmi-1 was related to the Lauren's and Borrmann's classification and the clinicopathological tumor stage, suggesting that Bmi-1 may be related to cell differentiation in the progression of gastric mucosa injury to cancerization. Therefore, Bmi-1 may underlie the tumorigenesis and infiltration of GC. Variation of Bmi-1 expression in intestinal and diffuse GC indicates that Bmi-1 may be related to cell differentiation, which is consistent with the over-expression of Bmi-1 in gastric IM. Liu *et al*[8] reported that Bmi-1 is up-regulated at

Table 3 Correlation between ki-67 and Bcl-2 expression and clinicopathological features of GC (Fisher's exact test)

Group	*n*	ki-67 expression +-+++(%)	χ^2	*P*	Bcl-2 expression +-+++(%)	χ^2	*P*
Gender			0.584	0.445		0.558	0.455
Male	116	98 (84.5)			68 (58.6)		
Female	46	41 (89.1)			24 (52.2)		
Age (yr)			0.004	0.950		0.206	0.650
≤ 60	82	71 (86.6)			48 (58.5)		
> 60	80	69 (86.3)			44 (55.0)		
Clinicopathological classification				0.080		1.464	0.226
EGC	30	29 (96.7)			20 (66.7)		
AGC	132	111 (84.1)			72 (54.5)		
Gross classification							
EGC				0.367			1.000
Ⅰ + Ⅱc	19	19 (100.0)			13 (68.4)		
Ⅲ	11	10 (90.9)			7 (63.6)		
AGC			13.380	< 0.001		2.632	0.105
Bor Ⅰ + Ⅱ	25	15 (60.0)			10 (40.0)		
Bor Ⅲ + Ⅳ	107	96 (89.7)			62 (57.9)		
WHO's histological classification			4.262	0.601		8.640	0.169
PA	4	4 (100.0)			3 (75.0)		
WDA	12	11 (91.7)			8 (66.7)		
MDA	50	45 (90.0)		1.000[a]	31(62.0)		1.000[a]
PDA	75	63 (84.0)		0.684[b]	35 (46.7)		0.229[b]
SRC	10	8 (80.0)			6 (60.0)		
MA	9	8 (88.9)			8 (88.9)		
UA	2	1 (50.0)			1 (50.0)		
Lauren's classification			2.146	0.143		5.552	0.018
Intestinal type	75	68 (90.7)			50 (65.8)		
Diffuse type	87	72 (82.8)			42 (48.8)		
Lymph node metastasis			0.021	0.885		0.045	0.832
No	98	85 (86.7)			55 (56.1)		
Yes	64	55 (85.9)			37 (57.8)		

Fisher's exact test, [a]*P* = 1.000, 1.000 *vs* PDA; [b]*P* = 0.684, 0.229 *vs* PDA.

Table 4 Relation between expressions of Bmi-1, ki-67 and Bcl-2 in GC and IM

	Bmi-1 in GC			r_k	*P*		Bmi-1 in IM			r_k	*P*
	-	+					-	+			
ki-67						ki-67					
-	14	8	22	0.123	0.119	-	4	6	10	0.177	0.199
+	64	76	140			+	13	59	72		
Bcl-2				0.157	0.043	Bcl-2				0.270	0.038
-	40	30	70			-	8	12	20		
+	38	54	92			+	9	53	62		
Total	78	84	162			Total	17	65	82		

both transcriptional and translational levels in GC tissues compared with that in its adjacent non-cancerous tissues, as confirmed by reverse transcription polymerase chain reaction and Western blotting, showing that Bmi-1 can serve as a valuable marker for the diagnosis and prognosis of GC.

Ki-67 is a nuclear antigen expressed in proliferating but not in quiescent cells. Consequently, ki-67 is used in tumor pathology to detect proliferating cells in neoplastic diseases. Bcl-2, known as a key regulator of the apoptosis, is a proto-oncogene first discovered in human follicular lymphoma and is involved in the inhibition of apoptosis and the survival of a variety of cell types. The distribution of Bcl-2^+/ki-67^- cells in gastric pyloric glands and intestine crypts might be potential cell compartments involved in cancerization of the gastrointestinal tract. In our study, Bcl-2^+/ki-67^- were used as potential markers for gastric stem cells, immunohistochemical double-labeling staining was used to display the distribution of Bcl-2^+/ki-67^- cells in GC and precancerous lesions, which showed that the distribution of Bmi-1^+ cells was consistent with that of Bcl-2^+/ki-67^- cells, and that Bmi-1 expression in IM was positively correlated with that of Bcl-2, suggesting that the expression of Bmi-1 is closely related with gastric cancer cellular proliferation and apoptotic progression of gastric carcinogenesis.

Lessard *et al*[9] reported that Bmi-1 has an essential role in regulating the proliferative activity of both normal and leukemic stem cells. It has been shown that Bmi-1 is a key regulator of self-renewal in both normal and tumorigenic human solid tumor stem cells, including

several types of brain cancer[10] and breast carcinoma[11]. Dovey *et al*[12] showed that Bmi-1 is over-expressed in numerous epithelial tumors and plays a key role in lung adenocarcinoma, thus providing a clue to lung cancer cell origin and lung tumorigenesis. Thus far, the relation between Bmi-1 and stem cells of gastrointestinal tract still remains unclear. Reinisch *et al*[4] reported that Bmi-1 expression serves as a potential stem cell marker of the gastrointestinal tract, which also shows that Bmi-1 expression is correlated with gastrointestinal stem cells as well as numerous specialized cell types. These results indicate that Bmi-1 protein is involved in cellular differentiation in addition to maintaining stem cells, which is consistent with the research of Molofsky *et al*[13]. Sangiorgi *et al*[14] found that Bmi-1 is expressed in discrete cells located near the bottom of crypts in small intestine. These cells proliferate, expand, self-renew and give rise to differentiated cell lineages of small intestinal epithelium, and ablation of Bmi1 (+) cells using a Rosa26 conditional allele expressing diphtheria toxin leads to crypt loss, suggesting that Bmi-1 is an intestinal stem cell marker *in vivo*.

In summary, Bmi-1 plays an important role in gastric cancer development, indicating that gastric cancer cells require Bmi-1 for their tumorigenic activity, and that interference with Bmi-1 activity may be a therapeutic strategy for GC. Thus, it is essential to elucidate the molecular mechanism of Bmi-1 involved in the cell cycle and to correlate this function with gastric stem cells in future.

COMMENTS

Background

It has been reported that B-cell-specific Moloney murine leukemia virus insertion site 1 (Bmi-1) is a transcriptional repressor that belongs to the polycomb-group family of proteins involved in hematopoiesis, regulation of proliferation and axial patterning. Bmi-1, an important factor for self-renewal and senescence of various stem cells, is highly expressed in various human malignant tumors.

Research frontiers

Bmi-1, identified as a protein that down-regulates $p16^{ink4a}$, is mandatory for the persistent existence of several stem cell classifications, such as hematopoietic and neural stem cells. It has been reported that Bmi-1 is a potential stem cell marker of the gastrointestinal tract. The expression of Bmi-1 is correlated with gastrointestinal stem cells as well as numerous other specialized cell types, and this protein plays a role in cellular differentiation rather than in stem cell maintenance. Bmi-1 is also a marker for carcinoma progression in nasopharyngeal cancer, bronchial carcinogenesis and myelodysplastic syndrome. Furthermore, microarray analyses performed in several other cancer types suggest that Bmi-1 mRNA is a prognostic marker.

Innovations and breakthroughs

In this study, immunohistochemical double-labeling staining was used to investigate the distribution of Bcl-2$^+$/ki-67$^-$ cells, and to explore its correlation with Bmi-1, which provides a valuable clue to the location of normal gastric mucosal and gastric cancer stem cells.

Applications

Investigating the expression of Bmi-1 in gastric carcinoma (GC) and precancerous lesions helps researchers analyze its role and significance in tumorigenesis of GC. Bmi-1 may serve as an adjuvant marker for the diagnosis and prognosis of GC.

Terminology

Bmi-1: an abbreviated form of B-cell-specific Moloney murine leukemia virus insertion site 1, a transcriptional repressor belonging to the polycomb group gene family.

Peer review

The study seems to be very interesting. The results, based on immunohistochemical observation, suggest that Bmi-1 plays a role in the progression of GC and is related to cell differentiation in the progression of gastricl mucosa injury to cancerization. Therefore, Bmi-1 may be used as an adjuvant prognostic marker. If the research incorporated reverse transcription polymerase chain reaction and Western blotting to quantify the RNA/protein expression, the results would be perfect.

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BRIEF ARTICLE

Efficacy of β-adrenergic blocker plus 5-isosorbide mononitrate and endoscopic band ligation for prophylaxis of esophageal variceal rebleeding: A meta-analysis

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Author contributions: Ding SH designed the study and wrote the manuscript; Wang JP collected the data; Liu J analyzed the available data and assessed the methodological quality of each study in accordance with the criteria by Jadad.

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Abstract

AIM: To systematically assess the efficacy and safety of β-adrenergic blocker plus 5-isosorbide mononitrate (BB + ISMN) and endoscopic band ligation (EBL) on prophylaxis of esophageal variceal rebleeding.

METHODS: Randomized controlled trials (RCTs) comparing the efficacy and safety of BB + ISMN and EBL on prophylaxis of esophageal variceal rebleeding were gathered from Medline, Embase, Cochrane Controlled Trial Registry and China Biological Medicine database between January 1980 and August 2007. Data from five trials were extracted and pooled. The analyses of the available data using the Revman 4.2 software were based on the intention-to-treat principle.

RESULTS: Four RCTs met the inclusion criteria. In comparison with BB + ISMN with EBL in prophylaxis of esophageal variceal rebleeding, there was no significant difference in the rate of rebleeding [relative risk (RR), 0.79; 95% CI: 0.62-1.00; $P = 0.05$], bleeding-related mortality (RR, 0.76; 95% CI: 0.31-1.42; $P = 0.40$), overall mortality (RR, 0.81; 95% CI: 0.61-1.08; $P = 0.15$) and complications (RR, 1.26; 95% CI: 0.93-1.70; $P = 0.13$).

CONCLUSION: In the prevention of esophageal variceal rebleeding, BB + ISMN are as effective as EBL. There are few complications with the two treatment modalities. Both BB + ISMN and EBL would be considered as the first-line therapy in the prevention of esophageal variceal rebleeding.

Key words: Meta-analysis; Esophageal variceal rebleeding; Endoscopic band ligation; β-adrenergic blocker; 5-isosorbide mononitrate; Prophylaxis

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INTRODUCTION

Cirrhotic patients with esophageal variceal bleeding have a very high incidence of rebleeding and a significant risk of death. Therefore, it was radical to adopt some interventional measures to prevent esophageal variceal rebleeding. Both endoscopic band ligation (EBL) and β-adrenergic blocker (BB) are the main therapies for secondary prophylaxis of esophageal variceal bleeding. Compared with untreated controls, these treatments can decrease the rate of variceal rebleeding and mortality[1-2]. Despite using adequate BBs, the portal pressure does not decrease in over one-third of patients[3]. Combined β-adrenergic blocker and 5-isosorbide mononitrate (BB + ISMN) was more effective than BBs alone in the prevention of esophageal variceal rebleeding[4-5]. It is still unknown whether drug therapy is superior to EBL for preventing variceal rebleeding. Several randomized controlled trials have shown different results[6-9].

Meta-analyses can statistically combine the results of several studies and resolve discrepancies among single studies. Because of combining the sample of individual studies, a meta-analysis greatly increases the overall sample size, which increases the statistical power of the

analysis, as well as the precision of the estimation of the therapeutic effect. The purpose of this study was to perform a meta-analysis of randomized controlled trials (RCTs) comparing BB + ISMN with EBL for secondary prophylaxis of esophageal variceal bleeding, and draw an overall conclusion about the safety and efficacy of the two treatments.

MATERIALS AND METHODS

Study selection

Any studies that met all of the following inclusion criteria were included: (1) the study was an RCT comparing the efficacy and safety of BB + ISMN and EBL on prophylaxis of esophageal variceal rebleeding; (2) duration of follow-up was at least 6 mo; and (3) outcome evaluation included at least one of the following: rebleeding, all-cause mortality, bleeding-related deaths and complications.

Search strategy

Medline, Embase, Cochrane Controlled Trial Registry and China Biological Medicine database were searched from January 1980 to August 2007 to locate published research in the area of esophageal variceal rebleeding. Key words used for searching included: esophageal variceal bleeding, BB, EBL, 5-ISMN, rebleeding, prevention and RCT. There was no language restriction applied to the search.

Assessment of study quality

Two of us independently assessed the methodological quality of each study in accordance with the criteria of Moher *et al*[10]. The trials were considered of high quality if the methodological quality score was three or more. The Jadad standard included four components: allocation sequence generation (computer-generated random number or similar, 2; not described, 1; and inadequate, 0); allocation concealment (central randomization and sealed envelops, 2; not described, 1; inadequate, 0); double blinding (identical placebo tablets or double dummy, 2; double blind but method not described, 1; no double blinding or inadequate method, 0); and description of protocol deviations, withdrawals and dropouts (numbers and reasons described, 1; not described, 0).

Statistical analysis

The measurement of association used in this meta-analysis was relative risk (RR) with 95% CI. Statistical heterogeneity between trials was evaluated by the Cochran Chi-square test and defined at a P value less than 0.1. In the absence of statistically significant heterogeneity, summary RR with 95% CI was calculated using fixed-effect models whereas potential reasons for heterogeneity was explored by subgroup analysis and sensitivity analysis using random-effect model. P value less than 0.05 was considered significantly different. All analyses and calculations were performed using the Revman 4.2 software.

RESULTS

Description of selected trials

Five RCTs met the inclusion criteria after searching the electronic databases, and one was excluded because it did not provide the same data. Four RCTs included 476 patients. The characteristics and quality of these four RCTs are summarized in Table 1. Two RCTs showed that BB + ISMN were as effective as EBL, one showed that pharmaceutical therapy was better, and the other showed a benefit of EBL. Three studies compared nadolol plus 5-ISMN with EBL, and propranolol plus 5-ISMN were administered in one study. A few patients in the EBL group received one or two sessions of sclerotherapy simultaneously in the Romero 2006 study.

Outcome evaluation

Rebleeding: Data from four randomized trials included 476 patients available for the assessment of rebleeding. Rebleeding was seen in 105 of 240 patients in the BB + ISMN group and in 109 of 236 patients in the EBL group. Summary RR for all four trials showed no significant difference in the rate of rebleeding between the BB + ISMN and EBL groups (RR, 0.94; 95% CI: 0.64-1.38; $P = 0.76$) using a randomized-effect model (Figure 1A). Test of heterogeneity for the rate of rebleeding was significant ($\chi^2 = 10.54$, $P = 0.01$). Clinical parameters were used to explore the cause of statistical heterogeneity. The proportion of patients who had large varices was higher in the BB + ISMN (30/61) than in the EBL group (19/60) in the LO2002 study[7]. Excluding this trial, the heterogeneity of χ^2 value for the remaining three trials was 2.37, $P = 0.31$. Summary RR for all these three trials showed no significant difference in the rate of rebleeding between the BB + ISMN and EBL groups (RR, 0.79; 95% CI: 0.62-1.00; $P = 0.05$) using a fixed-effect model.

All-cause mortality: Fifty-nine patients died in the BB + ISMN group and 72 in the EBL group. There was no significant heterogeneity between the studies ($P = 0.58$). Summary RR for all four trials showed no significant difference in the rate of all-cause mortality between the BB + ISMN and EBL groups (RR, 0.81; 95% CI: 0.61-1.08; $P = 0.15$) using a fixed-effect model (Figure 1B).

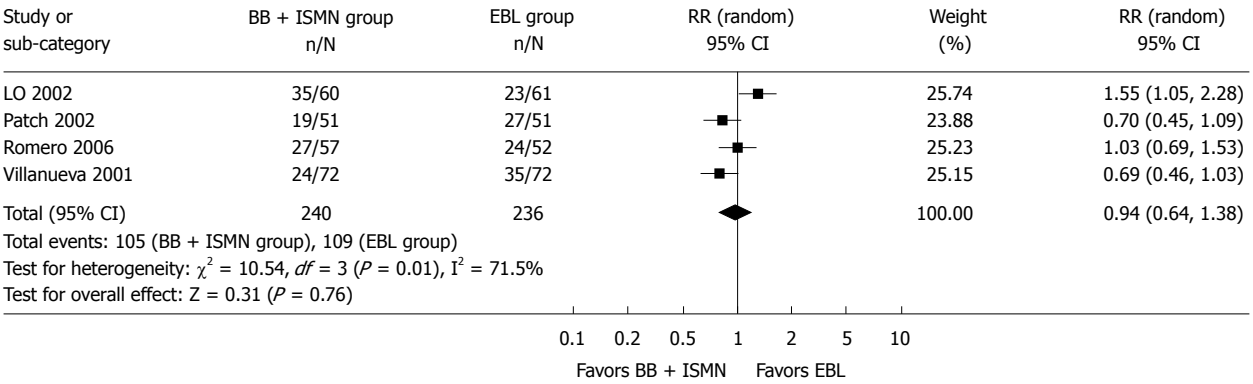
Bleeding-related deaths: Three trials evaluated bleeding-related deaths. There was no significant heterogeneity among studies ($P = 0.58$) and no significant difference in the rate of bleeding-related deaths between the BB + ISMN and EBL groups (RR, 0.76; 95% CI: 0.31-1.42; $P = 0.40$) (Figure 1C).

Complications: Adverse events were found in 76 patients in the BB + ISMN group including bradycardia, hypotension and headache, and 55 patients in the EBL group including bleeding ulcers, perforation, stenosis and chest pain. There was no mortality resulting from complications in either group. Summary RR for all four trials showed no significant difference in the occurrence of

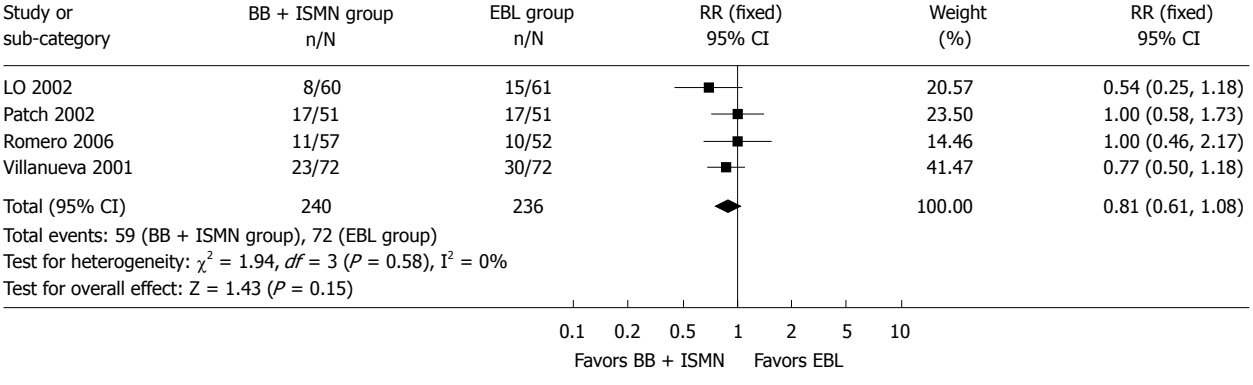
Table 1 Patient characteristics and Jadad score of included trials

Trials	Number of patients (BB + I/EBL)	Mean age (yr) (BB + I/EBL)	Males (BB + I/EBL)	Follow-up duration (BB + I/EBL)	Child-Pugh (A:B:C) (BB + I/EBL)	EBL mean sessions	BB + ISMN (mg/d)	Jadad score
Romero 2006	57/52	51 ± 10/53 ± 10	37:20/35:17	12/11.5 mo	23:25:9/ 17:30:5	3.4 ± 1.2	Nadolol 88 ± 68 5-ISMN 57.7 ± 27	6
PATCH 2002	51/51	50.7 ± 13.2/ 52.4 ± 13.4	35:16/35:16	248/356 d	8:19:24/ 5:18:28	2	Pronolol 80 (40-240) 5-ISMN	5
LO 2002	61/60	51 ± 13/52 ± 12	47:14/46:14	24/25 mo	13:35:13/ 13:35:12	3.3 ± 1.1	Nadolol 48 ± 10 5-ISMN 30 ± 6	5
Villanueva 2001	72/72	60 ± 12/58 ± 14	43:29/47:25	20/22 mo	19:39:14/ 11:43:18	2.1	Nadolol 96 ± 56 5-ISMN 66 ± 22	6

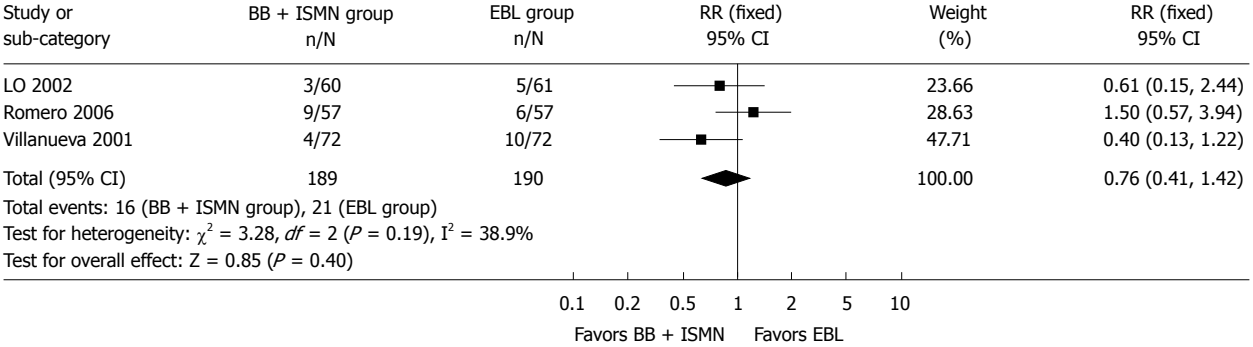
A Review: Prophylaxis of esophageal variceal rebleeding
Comparison: 01 BB + ISMN group *vs* EBL group
Outcome: 01 rebleeding rate



B Review: Prophylaxis of esophageal variceal rebleeding
Comparison: 01 BB + ISMN group *vs* EBL group
Outcome: 02 all-cause deaths



C Review: Prophylaxis of esophageal variceal rebleeding
Comparison: 01 BB + ISMN group *vs* EBL group
Outcome: 03 bleed-related deaths



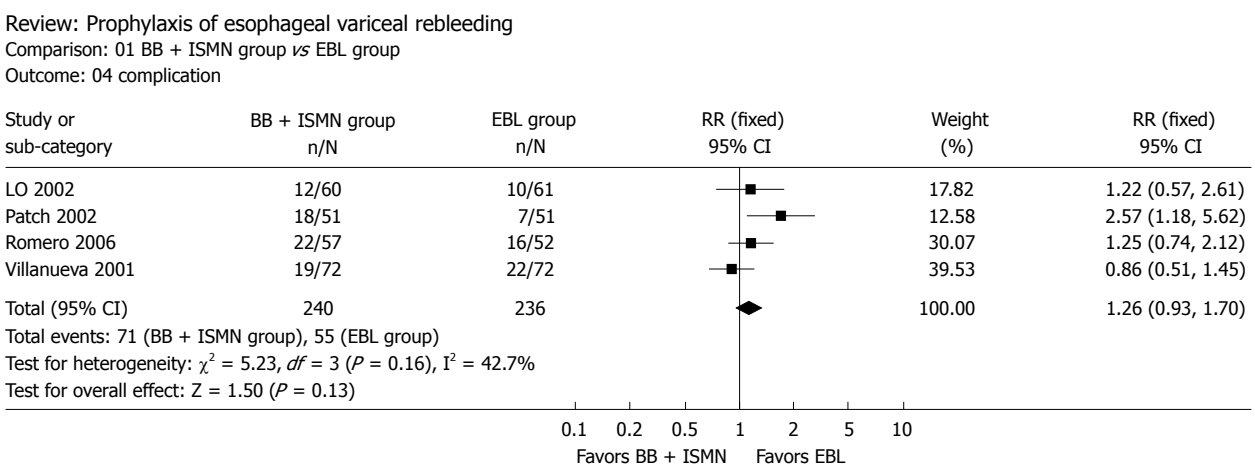


Figure 1 Comparison between BB + ISMN and EBL. A: Rebleeding rate; B: All-cause mortality; C: Bleeding-related mortality; D: Complication rate.

complications between the BB + ISMN and EBL groups (RR, 1.26; 95% CI: 0.93-1.70; $P = 0.13$) using a fixed-effect model (Figure 1D). Test of heterogeneity was not significant in the occurrence of complications ($P = 0.16$).

DISCUSSION

EBL has significantly reduced the frequency of variceal rebleeding, mortality and complications, and has replaced endoscopic injection sclerotherapy as the first-line therapy in the prevention of esophageal variceal rebleeding[11]. However, this treatment has a high recurrence, needs advanced technique and incurs a high cost[12]. The association of BB + ISMN enhances the reduction in portal pressure. Some clinical trials have found that combined BB + ISMN is superior to sclerotherapy and BB alone in the prevention of esophageal variceal rebleeding, with few complications, low cost and convenient administration[13]. It is still unknown whether drug therapy is superior to EBL for preventing variceal rebleeding. Our study included four RCTs and systematically assessed the efficacy and safety of BB + ISMN and EBL on prophylaxis of esophageal variceal rebleeding.

The meta-analysis showed that the overall rebleeding rate with BB + ISMN (43.8%) did not differ significantly from that of EBL (46.2%). There was a significant heterogeneity among the individual trials. The proportion of patients who had large varices was higher in the BB + ISMN (30/61) group than in the EBL group (19/60) in the LO2002 study, which may be the cause of the significant difference. Excluding this trial, there was no significant difference among the individual trials. Summary RR for all these three trials showed no significant difference in the rate of rebleeding between the BB + ISMN and EBL groups (RR, 0.79; 95% CI: 0.62-1.00; $P = 0.05$) using a fixed-effect model. The result showed that BB + ISMN was as effective as EBL in the prevention of esophageal variceal rebleeding. Decreasing the portal pressure by EBL did not result in increasing the bleeding at other local sites.

A total of 59 (24.5%) patients died in the BB + ISMN group and 72 (35.1%) in the EBL group. The mortality rate was similar in both groups (RR, 0.81; 95% CI: 0.61-1.08; $P = 0.15$). Three trials further evaluated the bleeding-related deaths, and there was no significant difference between the BB + ISMN and EBL groups (RR, 0.76; 95% CI: 0.31-1.42; $P = 0.40$). The comparative results between the BB + ISMN and EBL groups did not affect the all-cause and bleeding-related mortality.

Complications occurred in 71 (29.5%) patients in the BB + ISMN group and 55 (23.3%) in the EBL group ($P = 0.13$). None of the complications was fatal in either group. The occurrence rate of complications in our study was higher than that in other similar studies[14-15] because minor complications were included. Although Villanueva *et al*[6] showed that the incidence of severe adverse events was higher in the EBL group (12%) than in the BB + ISMN group (3%), this did not affect the overall result of our meta-analysis. However, the occurrence of complications was higher in the BB + ISMN group (29.5%) than in the EBL group (23.3%), and more patients withdrew from the study in the BB + ISMN group because they could not tolerate the complications of BBs.

In summary, combined therapy with BB + ISMN is as effective as EBL in the prevention of variceal rebleeding. The complications and survival are similar in the two interventional treatments. Both BB + ISMN and EBL are considered as the first-line therapy in the prevention of esophageal variceal rebleeding.

This meta-analysis was only based on published data and publication bias has not been evaluated because of the paucity of RCT data. The conclusion of this meta-analysis should be further demonstrated by large-scale and multicenter RCTs.

COMMENTS

Background

Cirrhotic patients who bleed from esophageal varices have a very high incidence of rebleeding and a significant risk of death. Both endoscopic band ligation (EBL) and β-adrenergic blocker plus 5-isosorbide mononitrate (BB + ISMN) are the main therapies for secondary prophylaxis of esophageal variceal bleeding. It is still unknown whether the drug therapy is superior to EBL for preventing variceal rebleeding. Several randomized controlled trials (RCTs)

have displayed different results. The authors performed a meta-analysis of RCTs comparing BB + ISMN with EBL for secondary prophylaxis of esophageal variceal bleeding, to draw an overall conclusion about the safety and efficacy of the two treatments.

Research frontiers

EBL has significantly reduced the frequency of variceal rebleeding, mortality and complications. However, this treatment has a higher recurrence, needs advanced techniques and is expensive. Some clinical trials have found that the combination of BB + ISMN is superior to sclerotherapy and BB alone in the prevention of esophageal variceal rebleeding, with few complications, and is cheap and convenient in administration.

Innovations and breakthroughs

To the best of our knowledge, this is the first published meta-analysis comparing BB + ISMN with EBL for secondary prophylaxis of esophageal variceal bleeding.

Applications

The research showed that combined therapy with BB + ISMN is as effective as EBL in the prevention of variceal rebleeding. BB + ISMN can be considered as the first-line therapy in the prevention of esophageal variceal rebleeding.

Peer review

Although good work has been done by this meta-analysis study, this paper needs some revisions.

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CASE REPORT

Unusual presentations of eosinophilic gastroenteritis: Case series and review of literature

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Abstract

Eosinophilic gastroenteritis (EG) is an uncommon disease characterized by focal or diffuse eosinophilic infiltration of the gastrointestinal tract, and is usually associated with dyspepsia, diarrhea and peripheral eosinophilia. Diffuse gastrointestinal tract and colonic involvement are uncommon. The endoscopic appearance may vary from normal to mucosal nodularity and ulceration. Gastrointestinal obstruction is unusual and is associated with predominantly muscular disease. We present five unusual cases of EG associated with gastric outlet and duodenal obstruction. Two cases presented with acute pancreatitis and one had a history of pancreatitis. Four cases responded well to medical therapy and one had recurrent gastric outlet obstruction that required surgery. Four out of the five cases had endoscopic and histological evidence of esophagitis and two had colitis. Two patients had ascites. These cases reaffirm that EG is a disorder with protean manifestations and may involve the entire gastrointestinal tract. Gastric outlet and/or small bowel obstruction is an important though uncommon presentation of EG. It may also present as esophagitis, gastritis with polypoid lesions, ulcers or erosions, colitis and pancreatitis and may mimic malignancy.

Key words: Gastroenteritis; Eosinophilic; Gastrointestinal obstruction; Eosinophilic esophagitis; Eosinophilic colitis; Eosinophilic pancreatitis

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INTRODUCTION

Eosinophilic gastroenteritis (EG) is an uncommon inflammatory disease characterized by eosinophilic infiltration of the gastrointestinal tract[1-5]. In 1937, Kaijser first described the disease in two patients with syphilis who were allergic to neoarsphenamine[5]. More than 300 cases have been reported in the literature since 1937[1-10]. The disease affects all races and any age group from infancy to old age, although in adults, it usually presents in the third to fifth decade[1-4].

It is reported to be more common in men with a ratio of 3:2[1-4]. Any part of the gastrointestinal tract from the esophagus to rectum may be involved. Eosinophilic proctocolitis is almost exclusively seen in children[1-4]. Although the exact etiology is unknown, a personal or family history of food allergies and atopic disorders can be elicited in 50% to 70% of cases[1-4]. Almost all patients have tissue eosinophilia; many have peripheral eosinophilia and raised IgE levels. The majority of cases have a favorable response to steroids, suggesting a type-1 hypersensitivity reaction. Eosinophils are bilobed granulocytes with secondary granules produced in the bone marrow under the influence of interleukin-3 (IL-3), IL-5 and granulocyte-macrophage colony-stimulating factor (GM-CSF)[1-4]. Eosinophils primarily reside in the

lining of the gastrointestinal tract providing protection against parasitic infections. The basic pathophysiological defect in EG is believed to be an alteration in the mucosal integrity, resulting in localization of various antigens in the gut wall and inducing tissue and blood eosinophilia[1-4,10,11]. Specific food antigens can cause mast cell degranulation in the gastrointestinal wall, releasing eosinophil chemotactic factors, leukotrienes and other platelet activating factors[12-16]. The degranulation of eosinophils causes the release of histamine, cationic proteins like major basic protein, eosinophil peroxidase, eosinophil-derived neurotoxin and cytokines such as tumor necrosis factor-α. Cytokines like GM-CSF, IL-3 and IL-5 induce eosinophil proliferation and differentiation in the bone marrow, and are strong chemotactic agents that attract eosinophils to sites of tissue inflammation[13-16]. These proteins promote inflammation, tissue damage and further mast cell degranulation, resulting in a vicious circle[10-14]. Eotaxin, a novel 73-amino-acid chemokine, plays a central role in the recruitment of eosinophils into tissues[10-16]. Eotaxin is a specific eosinophil chemoattractant produced by epithelial cells at the site of inflammation. It induces aggregation of eosinophils and promotes their adhesion to endothelial cells[2,9,15]. Some cases of EG are associated with unrecognized parasitic infestations and allergic or toxic reactions to drugs. An outbreak of eosinophilic enterocolitis due to the canine hookworm *Ankylostoma canium* was reported in Queensland, Australia[17,18]. Drugs such as gold, azathioprine, carbamazepine, enalapril, clofazimine and co-trimoxazole have been reported to cause eosinophilia with variable involvement of the gastrointestinal tract[19,20]. The clinical presentations of EG are protean[1-5,10] and may vary depending on the location and depth of involvement of the different layers of the digestive tract. On the basis of predominant involvement, Talley *et al*[7] and Klein *et al*[21] have classified eosinophilic gastroenteritis into mucosal, submucosal (muscular) and serosal disease. Mucosal disease is the most common (25%-100%) and presents with nausea, vomiting, abdominal pain, diarrhea and weight loss[1-5]. Muscular disease is the next most common (13%-70%) and presents with intermittent obstructive symptoms and complications such as perforation or aspiration. Serosal disease is less common (12%-40%). Intense peripheral eosinophilia, eosinophilic ascites and prompt response to steroid therapy are the hallmarks of serosal disease[1-7]. Rarely, EG may involve the pleura, pericardium, urinary bladder, pancreas, gall bladder, spleen, liver and the biliary tree[1-5,9,10].

The diagnosis is established by demonstrating eosinophilic infiltration on biopsies obtained on endoscopy, laparoscopy or laparotomy. Multiple biopsies are required because of the patchy nature of the disease[1,5,8,11]. Full-thickness surgical biopsies may be required for accurate diagnosis, if the disease process is confined to the muscle layer. An enzyme-linked immunosorbent assay has been developed in Australia to diagnose *Ankylostoma canium* infestation[17,18]. Barium studies, CT scanning and ultrasonography may all reveal thickening of the mucosal folds with or without nodular filling defects or gastric outlet obstruction. The CT scan may also demonstrate ascites, pleural effusions and lymphadenopathy in some cases[7,8,20]. The endoscopic findings may be patchy and vary from normal mucosa to mild erythema, thickened mucosal folds, nodularity and frank ulceration[1,5,7-9]. Corticosteroids remain the mainstay of treatment for EG. Some patients may have a relapsing course that requires long courses of steroid therapy.

CASE REPORT

Case 1

A 71-year-old woman presented with a history of nausea, abdominal pain, a weight loss of 10 pounds and diarrhea for 2 years. Stools studies were negative for ova, parasites and common pathogens. Clinical examination was unremarkable except for upper abdominal tenderness. A complete blood count revealed a WBC count of 6000/mm^3 with an eosinophil count of 8.2% (normal 0% to 4%). Other laboratory tests were unremarkable. The serum IgE level was 26 U/mL (normal 6-12 U/mL) and RAST testing for a battery of allergens, including common foods, was negative. CT scan of the abdomen was normal. Esophagogastroduodenoscopy (EGD) revealed distal esophagitis. There were multiple polypoid nodules in the gastric antrum, varying from 0.5 to 1 cm in size (Figure 1). Thickened gastric mucosal folds, antral erythema with small ulcers and erythema of the duodenal bulb were also noted. Histological examination of the polypoid nodules and biopsies from the esophagus, gastric antrum and duodenum demonstrated heavy eosinophilic infiltration and numerous degranulated eosinophils (Figure 2). Colonoscopy and random biopsies from the colon were normal. The patient was treated with prednisone 40 mg/d for 6 wk and tapered down to a maintenance dose of 10 mg/d for 6 mo, without much improvement in her symptoms. Repeat EGD revealed healing of the antral ulcers, without any change in the size and endoscopic appearance of the gastric polypoid lesions. Repeat biopsies revealed eosinophilic infiltration as before, with more fibrosis. Sodium cromoglycate 200 mg *tid* was added to her treatment with modest improvement of her symptoms. However, her abdominal pain recurred and she reported worsening nausea, postprandial fullness and bloating over the next 6 mo. Endoscopic examination revealed an increase in the number and size of the polypoid lesions, especially in the antrum, causing partial gastric outlet obstruction. Histological examination of the polypoid lesions demonstrated marked fibrosis but significantly decreased eosinophilic infiltration. Her obstructive symptoms worsened requiring antrectomy and gastrojejunostomy. She did well after surgery on low-dose steroids and sodium cromoglycate.

Case 2

A 57-year-old man presented with a history of generalized aches, nausea and upper abdominal pain for 4 mo. He was treated with H-2 blockers and later switched to proton pump inhibitors. His symptoms worsened and he developed postprandial fullness and bloating. His past medical history was remarkable for an episode of

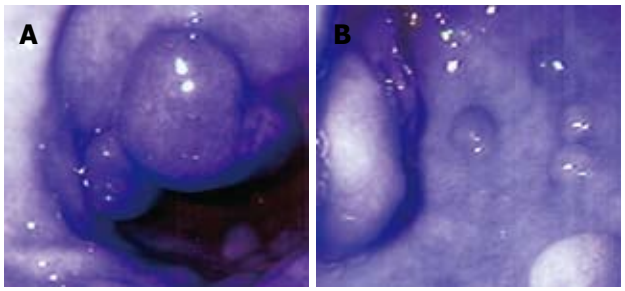

Figure 1 Endoscopic appearance of stomach (A and B) showing multiple gastric antral polyps of 4-10 mm in size and antral mucosal erythema.

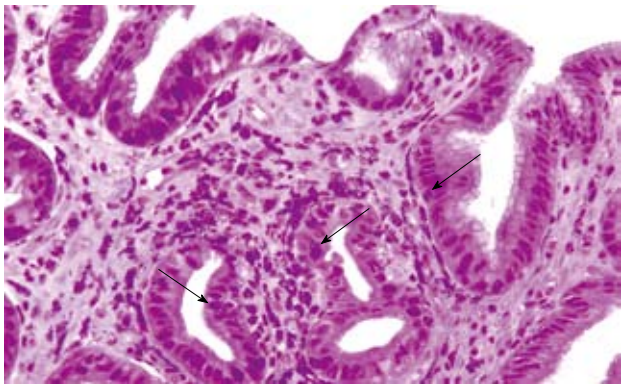

Figure 2 Histological appearance of the gastric polyp showing eosinophilic infiltration of the lamina propria by numerous degranulated eosinophils and some polymorphonuclear cells (arrows). Some intraepithelial eosinophils are also seen. (HE, × 200).

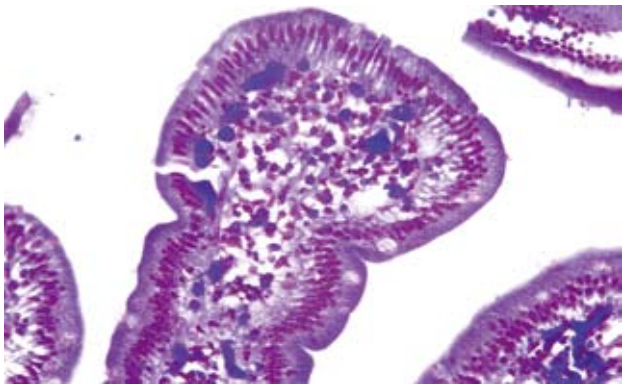

Figure 3 Histological appearance of duodenal mucosal biopsy demonstrating moderately severe infiltration with eosinophils and some intraepithelial eosinophils. (HE, × 200).

Figure 4 Barium upper GI demonstrating stricture and stenosis of the second and third part of duodenum, with reflux of the contrast medium into the biliary tree.

self-limiting pancreatitis of unclear etiology. He had no personal or family history of allergic disorders. Clinical examination demonstrated mild abdominal distension and epigastric tenderness. Laboratory data revealed, a WBC count of 10000/mm^3, and an eosinophil count of 33% (normal 0%-1%). The serum amylase level was 94 U/L (normal 25-115 U/L) and serum lipase was 415 U/L (normal 114-286 U/L). Stool studies for ova and parasites were negative. Barium X-ray series of the upper gastrointestinal tract revealed retained gastric secretions and narrowing of the gastric outlet with features of gastric outlet obstruction. Endoscopic examination demonstrated thickened and erythematous antral and duodenal folds with pyloric channel and duodenal narrowing. The gastric and duodenal biopsies revealed subacute and chronic inflammation with moderately intense eosinophilic infiltration (Figure 3). A CT scan of the abdomen demonstrated thickened pyloric and duodenal folds and unremarkable pancreas. The patient responded well to a course of oral steroids and his symptoms continued to improve on maintenance steroids.

Case 3

A 74-year-old man presented with intermittent bloating and fullness after meals for 3 years. He also complained of intermittent nausea and vomiting, abdominal pain and diarrhea. His symptoms had worsened over the past year and he had lost 10 pounds in weight. His past medical history was remarkable for an attack of pancreatitis of unclear etiology. He had no personal or family history of allergic disorders. Clinical examination was unremarkable except for mildly distended and tender abdomen. Laboratory tests revealed a WBC count of 6000/mm^3, and an eosinophil count of 9.6% (normal 0% to 4%). Serum IgE level was 54 U/mL (normal 6-12 U/mL). Stools studies were negative for ova and parasites. Barium X-rays of the upper gastrointestinal tract showed features of gastric outlet obstruction and irregular narrowing at the level of the second and third portion of the duodenum, with contrast medium refluxing into the common bile duct (Figure 4). A CT scan of the abdomen confirmed the presence of stenosis at the level of the second and third portion of the duodenum. An EGD revealed mild esophagitis and a dilated stomach and retained food. The antrum and proximal duodenal bulb were erythematous. There was mild narrowing at the level of the pylorus but significant narrowing of the duodenal bulb. The endoscope could not be advanced further. Biopsies from the antrum and the bulb showed moderately intense eosinophilic infiltration. Colonoscopy showed diverticular disease and random colonic biopsies revealed moderately intense eosinophilic infiltration. The patient did not accept steroid therapy or endoscopic dilatation initially. He had only partial improvement of symptoms with sodium cromoglycate 200 mg *tid*. His symptoms persisted and he

agreed to steroid therapy. He responded well to a 6-wk course of prednisone 40 mg/d, tapered down to a maintenance dose of 10 mg/d.

Case 4

A 43-year-old African American man was admitted with a history of intermittent abdominal pain, nausea, vomiting and diarrhea for 3 mo. He had experienced postprandial fullness and bloating for the past month and lost 8 pounds in weight. He had used ranitidine and omeprazole without benefit. He had no history of food allergies and his mild asthma was controlled by serevent inhalation. His clinical examination was remarkable for dehydration, abdominal distension and mild upper abdominal tenderness. Laboratory tests on admission revealed a WBC count of 6400/mm^3 with an eosinophil count of 20% (normal 0%-4%). Serum amylase was 471 U/L (normal 25-115 U/L) and serum lipase was 1785 U/L (normal 114-286 U/L). Serum IgE level was 650 U/L (normal < 140 U/L). Other laboratory tests including liver function tests, routine stool studies, serum lipid profile and serum immunoglobulins, were normal. An ultrasound and CT scan of the abdomen revealed a dilated stomach with retained food and mild thickening of the antral and duodenal folds. The pancreas was normal and there were no gallstones. The patient was treated conservatively with rehydration and nasogastric suction. EGD showed mild distal esophagitis, multiple antral erosions and thickened antral folds with antro-pyloric narrowing. Multiple 2-6-mm ulcerated nodules were noted in the duodenal bulb, with thickening of the duodenal folds extending into the second part of the duodenum. Biopsies of the esophagus revealed esophagitis with mild eosinophilic infiltration. Biopsies of the antrum and duodenum showed chronic gastritis and duodenitis with intense eosinophilic infiltration of the lamina propria and submucosa. Colonoscopy showed mild patchy erythema and biopsies showed mild eosinophilic infiltration in the lamina propria. The patient was treated with steroids. His eosinophil count normalized and his symptoms of gastric outlet obstruction resolved. His symptoms recurred on tapering down the steroids. Sodium cromoglycate was added to his therapy and helped in tapering down his steroids. After 6 mo of maintenance steroid therapy, he stopped the treatment and is doing well.

Case 5

A 60-year-old Indian woman was admitted with a history of upper abdominal pain for 3 wk, associated with nausea, vomiting and diarrhea. She had mild asthma controlled by albuterol inhalation. She was a teetotaller. Clinical examination was unremarkable except for mild upper abdominal tenderness. Laboratory data revealed a serum amylase of 375 U/L (normal 25-115 U/L) and serum lipase of 1115 U/L (normal 114-286 U/L). Complete blood count was remarkable for a WBC count of 12 500/mm^3 and an eosinophil count of 17% (normal 0%-4%). Other laboratory data including liver function tests, routine stool studies, serum lipid profile and serum immunoglobulins were normal. A CT scan of the abdomen was unremarkable. The patient was treated conservatively for a clinical diagnosis of acute idiopathic pancreatitis. However, she continued to have abdominal pain and diarrhea and lost about 20 pounds in weight over the next 5 wk. An endoscopic retrograde cholangiopancreatographic examination was normal except for prominent and erythematous ampulla. Brushings from the intra-ampullary pancreatic duct were normal and biopsies of the ampulla revealed dense eosinophilic infiltration with mild reactive glandular proliferation. An EGD showed distal esophagitis, antral gastritis and duodenitis with narrowing of the antrum and duodenal bulb. Biopsies revealed chronic esophagitis and gastritis with moderate eosinophilic infiltration, and severe chronic duodenitis with intense eosinophilic infiltration. The patient was treated with a course of steroids and responded promptly with resolution of symptoms and weight gain. She was maintained on a low dose of maintenance steroids for 3 mo. She was subsequently tapered off the steroids and is doing well.

DISCUSSION

These five patients presented had unusual manifestations of EG, testifying to the varied presentations of this disorder[1-5,7-9]. Two of five patients (40%) had a significant personal history of allergic disorders (asthma) and none had a history of food allergy. In a review of 220 cases, Naylor reported a history of allergy in 52% of cases[8]. Food allergy has been reported to be present in 50% of cases[1-5,21,22]. All five of our cases had abdominal pain and diarrhea, which are the most common symptoms in patients with EG being present in 72% and 50% of cases, respectively[1-5,7-9]. All five of our cases had predominant involvement of the stomach and duodenum, resulting in gastric outlet and duodenal obstruction. In the past, benign diseases such as peptic ulcer disease accounted for most of the cases of gastric outlet obstruction. With the evolution of effective therapy for peptic ulcer disease, malignancy and EG have emerged to be the most important causes of gastro-duodenal obstruction[23-26]. It is therefore imperative to rule out EG and malignancy in these patients. In children, EG may mimic congenital pyloric stenosis[24]. Other uncommon causes of gastric outlet obstruction including Crohn's disease, post-surgical strictures, pancreatic pseudocyst, gallstones and chronic pancreatitis should also be considered in the differential diagnosis. Weight loss, especially in elderly patients, should heighten the suspicion for malignancy. A long history of symptoms, unremarkable CT scan and normal tumor markers may be helpful in ruling out a malignant etiology.

All five of our cases (100%) had endoscopic and histological evidence of eosinophilic gastritis and duodenitis. Endoscopic and histological evidence of eosinophilic esophagitis was present in four of our five (80%) cases and one patient did not have esophageal biopsies performed. Colonoscopy and random colonic biopsies were performed in three cases and revealed eosinophilic colitis in two (66%). Although EG can involve the entire gastrointestinal tract, the esophagus

and colon are uncommonly involved[1-5,26]. However, esophageal involvement is now more frequently reported, especially in children and young adults[1-5].

In a review of 220 cases, Naylor *et al*[8] reported that the stomach was the most frequently involved organ (43% of cases). The duodenum and the rest of the small bowel are less frequently involved. Small bowel involvement may present with abdominal pain, diarrhea or frank malabsorption[27,28], and rarely bowel obstruction[1-5,7-9,29-31]. There have been only a few case reports of jejunal and ileal strictures[25,26]. Colonic involvement presents as abdominal pain and/or diarrhea[9,11,29-31].

Eosinophilic gastroenteritis may present as an acute abdomen due to acute pancreatitis, intestinal or colonic obstruction, intussusception and perforation[25,26,31-33]. Two of our five cases (40%) presented with acute pancreatitis of unknown etiology. Interestingly, the two cases with acute pancreatitis had very high eosinophil counts, and biopsies from a prominent and erythematous ampulla in one patient showed intense eosinophilic infiltration. There was no recurrence of pancreatitis after steroid treatment, supporting eosinophilic infiltration as the etiology. The barium X-rays of one of the patients with a history of pancreatitis revealed an interesting finding of spontaneous barium reflux into the biliary tree (Figure 4). We believe the duodenal stricture from EG facilitated the reflux of barium into the biliary tree. Eosinophilic infiltration can cause edema, fibrosis and distortion in the ampulla and periampullary duodenum and cause pancreatitis[33,34]. Pancreatic involvement may also mimic a pancreatic malignancy[33,34]. Hepatic, splenic, biliary tract, gall bladder and urinary bladder involvement has also been reported[1-5,7-9,34-37].

The peripheral eosinophil count was high in all five (100%) of our cases and very high in two cases. Peripheral eosinophilia has been reported in up to 80% of cases[1-5,7]. Patients with predominantly serosal disease have higher absolute eosinophil counts (average 8000/dL) than patients with mucosal disease (average 2000/dL) and muscle layer disease (average 1000/dL)[33,34]. None of our cases had evidence of significant blood loss or malabsorption, which have been reported in the literature in 20%-30% patients, especially those with mucosal disease[6-8]. Serum IgE level was checked in only three of our patients and was elevated in two (66%). IgE levels are more likely to be high in children with EG than in adults[35,36]. Our patients demonstrated almost the whole spectrum of endoscopic features including, erythema, ulcers, nodularity, thickening of folds and pseudopolypoid lesions.

All five of our patients (100%) responded to steroid therapy. Only one patient required surgery for gastric outlet obstruction. One of our patients had a partial response to sodium cromoglycate and in another patient this drug helped in tapering off his steroids. Steroids are the mainstay of treatment in EG and about 90% patients respond to this therapy[5,7-9]. Patients with serosal disease usually show a dramatic response to steroids[1-5,7-9]. Azathioprine may be helpful as a steroid-sparing agent in patients requiring high doses for maintenance. Sodium cromoglycate is a mast cell stabilizer that prevents release of toxic mediators like histamine, platelet activating factors and leukotrienes from mast cells. There have been several reports of a beneficial response to this drug[38,39]. The usual dose is 200 mg three or four times per day. Ketotifen is similar to sodium cromoglycate in its biological profile and may be useful in some cases[40]. Elimination of presumed dietary articles is unhelpful in most cases[1-5]. Successful treatment of EG with montelukast, a leukotriene modifier, has been reported[12]. Suplatast tosilate is a new IL-4 and IL-5 inhibitor effective in treating asthma, and has been reported to be useful in treating a patient with EG[41]. A humanized anti-IL-5 monoclonal antibody (mepolizumab) has been found to be beneficial in a small series of four patients with hypereosinophilia syndrome[42]. This antibody may have a potential therapeutic role in treating patients with EG.

As demonstrated by our five cases, the clinical course of EG is highly variable. However, the long duration of illness in most cases testifies to the generally good prognosis of EG. Fatalities from EG are rare and are usually due to perforation of the gastrointestinal tract[43,44].

From our experience with these five cases, we conclude that EG is truly protean in its clinical and endoscopic manifestations, sites of involvement in the digestive system, response to therapy and clinical course. Gastroduodenal involvement is common, esophageal involvement is being increasingly reported and colonic involvement is uncommon. EG can present with gastric outlet and duodenal stricturing resulting in gastric outlet obstruction. Malignancy is an important differential diagnosis and should be ruled out by appropriate diagnostic modalities. Patients with EG may present with acute pancreatitis and this should be considered in the differential diagnosis. The course of EG is variable and relapses are common. However, the response to treatment and overall prognosis is good.

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CASE REPORT

Endoscopic submucosal dissection of a rectal carcinoid tumor using grasping type scissors forceps

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Abstract

Endoscopic submucosal dissection (ESD) with a knife is a technically demanding procedure associated with a high complication rate. The shortcomings of this method are the inability to fix the knife to the target lesion, and compression of the lesion. These can lead to major complications such as perforation and bleeding. To reduce the risk of complications related to ESD, we developed a new grasping type scissors forceps (GSF), which can grasp and incise the targeted tissue using electrosurgical current. Colonoscopy on a 55-year-old woman revealed a 10-mm rectal submucosal nodule. The histological diagnosis of the specimen obtained by biopsy was carcinoid tumor. Endoscopic ultrasonography demonstrated a hypoechoic solid tumor limited to the submucosa without lymph node involvement. It was safely and accurately resected without unexpected incision by ESD using a GSF. No delayed hemorrhage or perforation occurred. Histological examination confirmed the carcinoid tumor was completely excised with negative resection margin.

Key words: Endoscopic submucosal dissection; New device; Rectal carcinoid; Grasping type scissors forceps; Endoscopic therapy

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INTRODUCTION

Recently, colonoscopy has facilitated the diagnosis of rectal carcinoid tumors at an early stage. Theoretically, rectal carcinoid tumors less than 1 cm in diameter and a depth of invasion limited to the submucosal layer can be curatively treated by endoscopic resection[1-4]. Conventional snare polypectomy or endoscopic mucosal resection often results in incomplete resection of rectal carcinoid tumors and the need for additional surgery that sometimes requires a stoma[4-6]. Endoscopic submucosal dissection (ESD) has been reported to improve the rate of successful *en bloc* resection in early stage rectal tumors[6]. Furthermore, ESD can accurately control the depth of submucosal exfoliation under endoscopic view. However, ESD, and particularly the process of submucosal dissection, is technically difficult and carries a high risk of perforation and bleeding[6-9]. Conventional devices for submucosal incision such as IT knife and needle knife merely contact the knife to the submucosal tissue and cut using electrosurgical current. These cutting methods without fixing the knife to the target have a potential risk of incomplete resections or major complications due to unexpected incision. To resolve the problems related to ESD using a conventional knife, we have developed a grasping type scissors forceps (GSF), which can accurately grasp and incise the targeted tissue using electrosurgical current[10,11]. In our previous study for early gastric neoplasms, we resected four tumors safely and easily without unintentional incision by ESD

using the GSF[9]. In this report, we first describe a new method of ESD using GSF for rectal carcinoid tumors.

CASE REPORT

Colonoscopy on a 55-year-old woman revealed a 10-mm rectal submucosal nodule (Figure 1A). The histological diagnosis of the specimen obtained by biopsy was carcinoid tumor. Subsequent endoscopic ultrasonography (EUS) demonstrated a hypoechoic solid tumor in the submucosa without lymph node involvement (Figure 1B). It was treated by ESD using a newly developed GSF (XDP2618DT; Fujifilm, Saitama, Japan) (Figure 2)[10], after obtaining written informed consent from the patient. A two-channel multi-bending endoscope (GIF-2T240M; Olympus, Tokyo, Japan) was used in this case. During ESD, the patient was sedated with an intravenous injection of flunitrazepam (0.4 mg) and pethidine (35 mg). The ESD technique using GSF was carried out as follows (Figure 3). Marking dots were placed approximately a few millimeters outside the margin of the lesion with a hook knife (KD-620LR; Olympus, Tokyo, Japan), with a coagulation current of 20 W (Forced coagulation mode) created by an electrosurgical generator (ICC 200; Erbe, Tübingen, Germany). Next, a concentrated glycerin solution mixed with a small volume of epinephrine and indigo carmine dye was injected into the submucosal layer around the target lesion to lift the entire lesion. The lesion was separated from the surrounding normal mucosa (Figure 4A) around the lesion with the GSF using an electrosurgical current (Autocut mode 120 W). A piece of submucosal tissue was grasped and cut with the GSF (Autocut mode 120 W) to achieve submucosal excision. During the dissection, ESD using GSF can accurately control the depth of submucosal excision under endoscopic vision (Figure 4B). Finally, the lesion was completely resected (*en bloc* resection) by GSF (Figure 4C). It took 91 min for the ESD. Macroscopically, the mass was yellowish-white and solid, measuring 11 mm × 10 mm in diameter. Microscopically, the tumor was composed of small uniform cells, arranged in small nests and cords, with an anastomosing ribbon-like pattern in the submucosal layer. Immunohistochemically, the tumor cells were positive for synaptophysin. The vertical and horizontal cut margins were negative. There was no lymphovascular invasion. These findings established curative resection of the rectal carcinoid tumor (Figure 4D). After ESD, the patient stayed in the hospital and was prohibited from eating until the fourth day of ESD. Laboratory findings and chest and abdominal X-ray remained unremarkable after ESD. She was permitted oral soft food and discharged 7 d after the procedure. No hemorrhage, perforation, or other complication occurred.

Newly developed GSF

The GSF (XDP2618DT) (Figure 2) can grasp and cut a piece of tissue, using an electrosurgical current. It has a 0.4-mm wide and 4-mm long serrated cutting edge to facilitate grasping the tissue. The outer side of the forceps is insulated so that electrosurgical current energy is concentrated at the blade to avoid burning the surrounding tissue. Furthermore, the forceps can be rotated to the desired orientation. The diameter of the forceps is 2.7 mm. The GSF is available for standard endoscopy with a working channel width of 2.8 mm or over. This device, which is disposable and not reusable, was used for circumferential marginal incision and submucosal dissection.

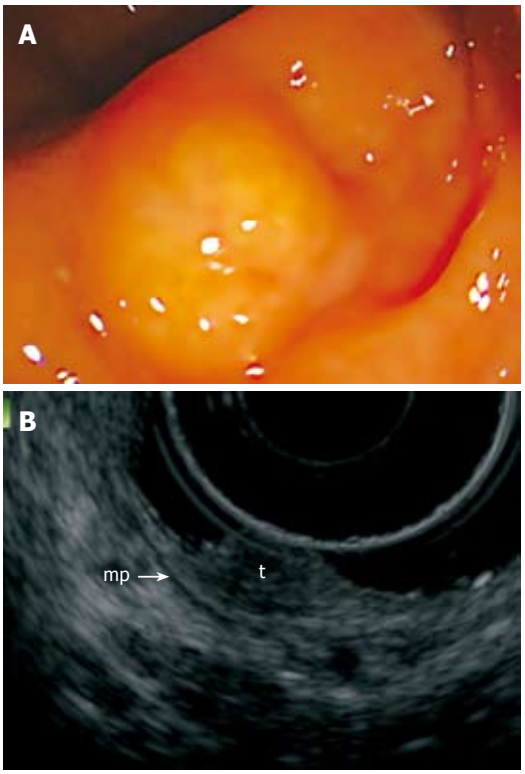

Figure 1 Pretherapeutic examinations of rectal carcinoid. A: Endoscopic view of the small rectal carcinoid; B: EUS showing a hypoechoic solid tumor (t) in the superficial submucosa. Arrow-mp: Muscularis propria.

Ethical considerations

The advantages and disadvantages of the ESD using GSF, as well as alternative endoscopic options (ESD using conventional device, endoscopic mucosal resection *etc*), were discussed with the patient. The patient was aware of the experimental nature of the planned treatment. She gave her written informed consent to the designated intervention. This study was reviewed and approved by the ethics committee of Aso Iizuka Hospital. It was conducted in accordance with the ethical principles of the Declaration of Helsinki and in compliance with good clinical practice.

DISCUSSION

The rectum is one of the most frequent primary sites of carcinoid tumors. Rectal carcinoids less than 2 cm rarely metastasize, indicating local excision including endoscopic resection[2]. Furthermore, when the tumor is smaller than 1 cm and the depth of invasion is lim-

Figure 2 Distal tip of the GSF. The outer side of the forceps is insulated so that electrosurgical current energy is concentrated at the blade to avoid burning the surrounding tissue.

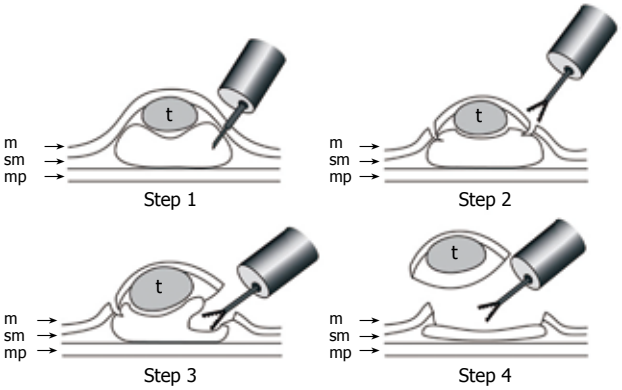

Figure 3 Schematic shows ESD using GSF. Step 1: A concentrated glycerin solution mixed with a small volume of epinephrine and indigo carmine dye is injected into the submucosal layer around the target lesion to lift the entire lesion; Step 2: The lesion is separated from the surrounding normal mucosa by complete incision around the lesion using the GSF; Step 3: A piece of submucosal tissue is grasped and cut with the forceps using an electrosurgical current to effect submucosal exfoliation; Step 4: The lesion is resected in one piece. m: Mucosa; sm: Submucosa; mp: Muscularis propria; t: Tumor.

ited within submucosa, the risk of metastatic disease is extremely low, and endoscopic resection is considered curative[1-5]. Technically, complete resection of carcinoid tumors of the rectum is difficult with conventional endoscopic polypectomy[12], because 76% of these tumors extend into the submucosa[4-6]. However, various modified endoscopic therapies, such as strip biopsy[13], aspiration resection[14], band-snare resection[15] and endosonography probe-guided band ligation[16] result in good outcome for submucosal rectal carcinoid tumors less than 1 cm, so the application of ESD for carcinoids may be limited. When the lesions are larger, around 1-2 cm (1.1 cm in our case), or massively invade the submucosal layer, which may result in tumor-positive margin resection[3], incomplete resection with endoscopic resection sometimes results in the need for additional surgery. In such circumstances, ESD should be applied[3].

ESD was originally developed to obtain one-piece resection for early gastric cancers[7-11]. ESD has the advantage of permitting *en bloc* resection and histologically complete resection. On the other hand, this method has the disadvantages of a long procedure time and a high frequency of complications, as well as demanding a high level of technical skill. However, ESD can control

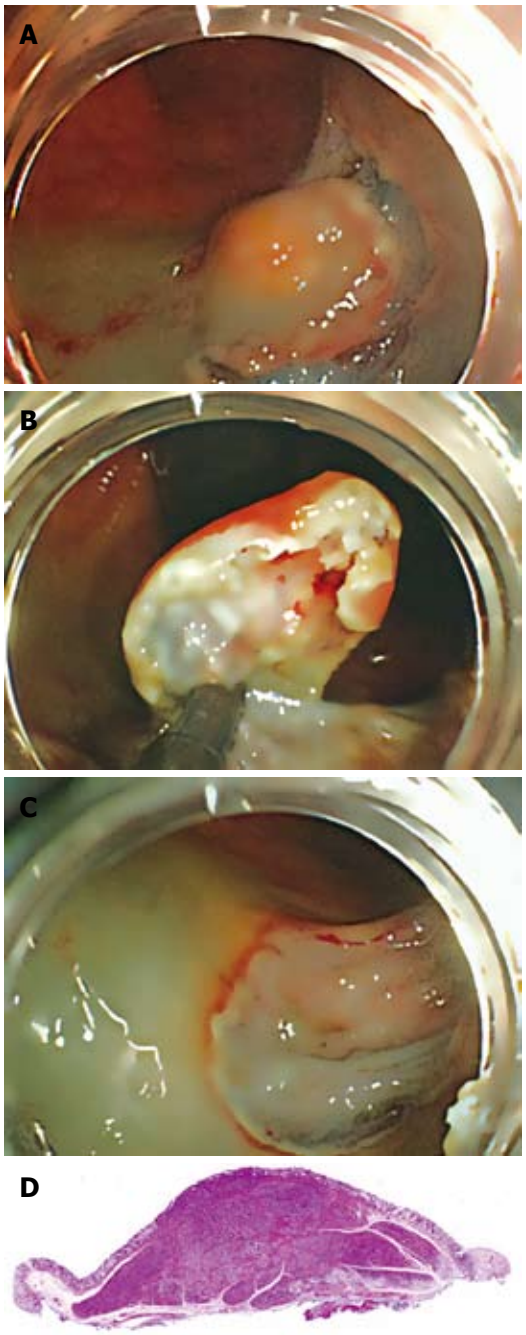

Figure 4 ESD using GSF of rectal carcinoid. A: Endoscopic view of the partial circumferential incision of the tumor using GSF; B: Endoscopic view of the submucosal exfoliation under the tumor using GSF; C: The lesion is cut completely from the muscle layer; D: The resected specimen showing curative *en bloc* resection of the lesion.

the depth of submucosal dissection under endoscopic view[6-11]. Therefore, ESD is a theoretically suitable therapeutic option for rectal carcinoid located within the submucosa[2,3,6]. If the tumor invades the muscularis propria, ESD is contraindicated due to the risk of perforation and metastasis. Pretherapeutic EUS is vital for decision making concerning the indication of ESD for this disease.

Incision using knife devices merely contact the knife to the tissue and cut using electrosurgical current. These cutting processes without fixing the device to the tar-

geted tissue make it difficult to place the knife accurately during electrosurgical incision because of bowel movement. Lack of complete endoscopy control can cause unexpected incision and result in incomplete resection or severe complications such as perforation and bleeding[6-9]. Our approach was to perform endoscopic resection with a GSF that can be passed through the ordinary working channel. This device was developed by us for ESD of early gastric cancer[10,11]. It has a thin serrated cutting edge to facilitate grasping the tissue. The outer side of the forceps is insulated so that electrosurgical current energy is concentrated at the blade to avoid burning the surrounding tissue. Furthermore, the forceps can be rotated to the desired orientation. Theoretically, the main advantage of GSF for ESD is the fixed device, which can accurately control the depth of submucosal exfoliation under good endoscopic vision[11]. GSF can be used to grasp the targeted tissue again if necessary, before electrosurgical cutting. Furthermore, the GSF can reduce post-cut hemorrhage by a compression effect similar to a polypectomy snare[11]. Thus the grasping step before cutting allows accurate targeting and compression of the vessel, and reduces the chance of incomplete resection and major complications (perforation and bleeding). In our method, sufficient separation of the tumor from the underlying muscularis propria, using submucosal injection of the solutions, is effective in preventing perforation due to thermal damage or capture of the muscularis propria by the GSF. Therefore, frequent additional submucosal injection of solution during the procedure is vital to reduce the risk of such complications. Each cut of the GSF goes a length of about 4 mm. As for the perforation, the direction of the device is the most important factor. If the device goes as far as the muscularis propria, perforation will occur, so we should operate the device parallel to the muscularis propria. The maximal advantage of the GSF is having the visual confirmation step for accurate and safe targeting by the device before cutting during the grasping stage. However, the GSF is unsuitable for marking like an IT-knife. For marking, a needle knife, flex knife, argon plasma coagulator probe, *etc* are available. In our method, we used a hook-knife. Furthermore, if the scope is strongly retroflexed, the rotation of the GSF is a little difficult. This is the limitation of this device. In this case, it was safe and accurate to resect the rectal carcinoid with a sufficient negative resection margin using GSF. To the best of our knowledge, this is the first report of ESD using GSF for rectal carcinoid. We believe this technique has the potential to become the method of choice for removal of GI tract carcinoid tumor when the tumor is limited to the submucosa.

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CASE REPORT

Lansoprazole-associated collagenous colitis: Diffuse mucosal cloudiness mimicking ulcerative colitis

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Abstract

There have only been a few reports on lansoprazole-associated collagenous colitis. Colonic mucosa of collagenous colitis is known to be endoscopically normal. We present a case of collagenous colitis where the mucosa showed diffuse cloudiness mimicking ulcerative colitis. A 70-year-old woman developed watery diarrhea four to nine times a day. She had interstitial pneumonia at 67 and reflux esophagitis at 70 years. Lansoprazole 30 mg/d had been prescribed for reflux esophagitis for nearly 6 mo. Lansoprazole was withdrawn due to its possible side effect of diarrhea. Colonoscopy disclosed diffuse cloudiness of the mucosa which suggested ulcerative colitis. Consequently sulfasalazine 2 g/d was started. The patient's diarrhea dramatically disappeared on the following day. However, biopsy specimens showed subepithelial collagenous thickening and infiltration of inflammatory cells in the lamina propria, confirming the diagnosis of collagenous colitis. One month after sulfasalazine therapy was initiated, colonoscopic and histological abnormalities resolved completely. Five months later the diarrhea recurred. The findings on colonoscopy and histology were the same as before, confirming a diagnosis of collagenous colitis relapse. We found that the patient had begun to take lansoprazole again 3 mo ahead of the recent diarrhea. Withdrawal of lansoprazole promptly resolved the diarrhea. Endoscopic and histological abnormalities were also completely resolved, similar to the first episode. Retrospectively, the date of commencement of sulfasalazine and discontinuation of lansoprazole in the first episode was found to be the same. We conclude that this patient had lansoprazole-associated collagenous colitis.

Key words: Collagenous colitis; Microscopic colitis; Lansoprazole; Ulcerative colitis; Sulfasalazine

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INTRODUCTION

Collagenous colitis and lymphocytic colitis, collectively termed microcytic colitis, are considered to be etiologically related and to be a spectrum of the same disease[1]. The disease is well known to have normal mucosa endoscopically. However, endoscopic abnormalities are observed in about 30% of cases: abnormal vascular pattern, loss of vascular pattern, edema, and erythema[2,3]. The treatment of the disease is similar to that in ulcerative colitis[3,4]. The etiology of the disease is unknown but a significant proportion may be drug-induced[5-7]. Immunological disposition is implicated because the disease is often seen in patients with a variety of autoimmune diseases[4,8].

Lansoprazole is widely prescribed for gastroesophageal reflux and benign peptic ulcer disease. We present a case of lansoprazole-associated collagenous colitis where the mucosa showed diffuse cloudiness mimicking ulcerative colitis. We mistook this case initially for an atypical case

of ulcerative colitis, and the patient was then diagnosed with collagenous colitis, which seemed to respond to sulfasalazine. In fact, the response was to the removal of lansoprazole.

CASE REPORT

A 70-year-old woman with watery diarrhea four to nine times a day in an orthopedic ward was referred to a gastroenterologist at the beginning of April 2007. She had a past history of: pulmonary tuberculosis and hypertension at 54 years, cerebral hemorrhage with a sequel of left hemiplegia at 63; diabetes mellitus, constipation, internal hemorrhoid and interstitial pneumonia at 67; neurogenic bladder at 68; gastroduodenal ulcers at 69; and reflux esophagitis at 70. She had a fracture of the femoral head and underwent surgery for insertion of an artificial femoral head on March 16, 2007. The patient had diarrhea four to nine times a day since March 26. Abnormalities on routine blood testing included mild anemia (hemoglobin 105 g/L), hypoproteinemia (49 g/L) and increased C-reactive protein (33 mg/L). Stool culture for pathogens was negative and fecal occult blood tests were negative. The following immunological and hormonal tests were normal: anti-nuclear antibody, rheumatic factor, perinuclear antineutrophil cytoplasmic antibody, anti-Scl 70 antibody, anti-centromere antibody, thyroid test, microsome antibody, free T3, free T4, and thyroid stimulating hormone. The gastroenterologist (MC) decided to check her drugs for diarrhea as a side effect and asked the orthopedist to withdraw lansoprazole 30 mg/d if possible, and to change loxoprofen sodium to etodolac. Lansoprazole had been prescribed for reflux esophagitis for nearly 6 mo and loxoprofen sodium had been prescribed for anal pain of unknown cause after the episode of diarrhea. Neither metronidazole of 1 wk duration for suspected antibiotic-associated diarrhea nor trimebutine maleate of 1 wk duration for suspected irritable bowel syndrome was effective. Therefore, colonoscopy was performed on April 16. This disclosed diffuse cloudiness of the mucosa in the entire colorectum observed from the rectum to the descending colon (Figure 1A). These findings suggested ulcerative colitis. Consequently, sulfasalazine 2 g/d was started that day. The patient's diarrhea dramatically disappeared on the following day. The findings of three biopsy specimens each from the descending colon, the sigmoid colon, and the rectum showed similar results: erosion and moderate infiltration of inflammatory cells in the lamina propria (Figure 2A). Crypt abscess was not found, however, subepithelial collagenous thickening was found (Figures 2A and 3A). Therefore, collagenous colitis was diagnosed. One month later, colonoscopy showed a clear vascular pattern (Figure 1B) and disappearance of subepithelial collagenous thickening (Figures 2B and 3B). Neither lansoprazole nor loxoprofen sodium was prescribed on her discharge (May 25, 2007). The dose of sulfasalazine was later decreased from 2 g/d to 1 g, followed by 0.5 g/d. Since diarrhea recurred around the end of September 2007, the dose of sulfasalazine was increased to 2 g/d. However, diarrhea persisted and she was readmitted on December 10, 2007. Since the findings on colonoscopy and histology were the same as before, a relapse of collagenous colitis was diagnosed. Following admission, we found that the patient had been taking lansoprazole since July 2, 2007 which was prescribed by another hospital. At this time, we were aware of lansoprazole-associated collagenous colitis[9-12]. Lansoprazole was withdrawn on December 16. The diarrhea improved within a few days. Endoscopic and histological normalization was ascertained on January 31, 2008. We retrospectively found that in the first episode, the date of withdrawal of lansoprazole by the orthopedist coincided with the commencement of sulfasalazine. We finally diagnosed this patient as having lansoprazole-associated collagenous colitis.

DISCUSSION

Microscopic colitis was originally described as mucosa that is endoscopically normal. Recently new endoscopic findings have been added: red spots[13], aphthoid ulcer[14], ulcer[6], mucosal tears[15,16], hemorrhagic lacerations[17], and longitudinal ulcers[18]. In our case, distinct diffuse cloudiness of the mucosa was observed on two occasions in this patient with collagenous colitis. In collagenous colitis, in addition to subepithelial collagenous thickening, there are significant numbers of inflammatory cells in the lamina propria. These changes completely disappear on recovery. Therefore, it seems reasonable that diffuse mucosal cloudiness rather than normal mucosa is endoscopically observed in collagenous colitis.

Diffuse cloudiness of the mucosa can be seen in a mild type of ulcerative colitis. Therefore, we at first diagnosed the patient with ulcerative colitis and sulfasalazine was prescribed. The current strategy for collagenous colitis is similar to that of ulcerative colitis[3,4]. In this patient, sulfasalazine seemed dramatically effective against ulcerative colitis before collagenous colitis was diagnosed, and against collagenous colitis after collagenous colitis was diagnosed.

Microscopic colitis is associated with a variety of immunological disorders and immunological phenomena: thyroid disease, rheumatoid arthritis, polyarthritis, CREST syndrome, eosinophilia, and the presence of autoantibodies[4,8]. The present case had interstitial pneumonia, which is frequently associated with autoimmune diseases. Interstitial pneumonia is to be added to a list of immunological disorders associated with microscopic colitis.

Microscopic colitis is known to be associated with various drugs including nonsteroidal anti-inflammatory drugs[5-7]. Recently, lansoprazole has been shown to cause microscopic colitis[9-12,18]. The frequency of lansoprazole-associated microscopic colitis is not known, but at least six of 850 subjects who took lansoprazole (0.7%) were found to develop microscopic colitis[10]. The period from

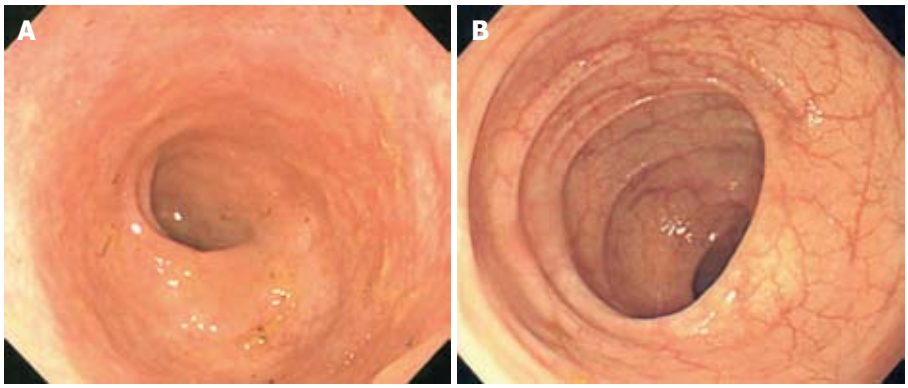

Figure 1 Colonoscopy on April 16 (A) and May 17 (B), 2007 showed diffuse cloudiness of mucosa in the colon and clear normal vascular patterns, respectively.

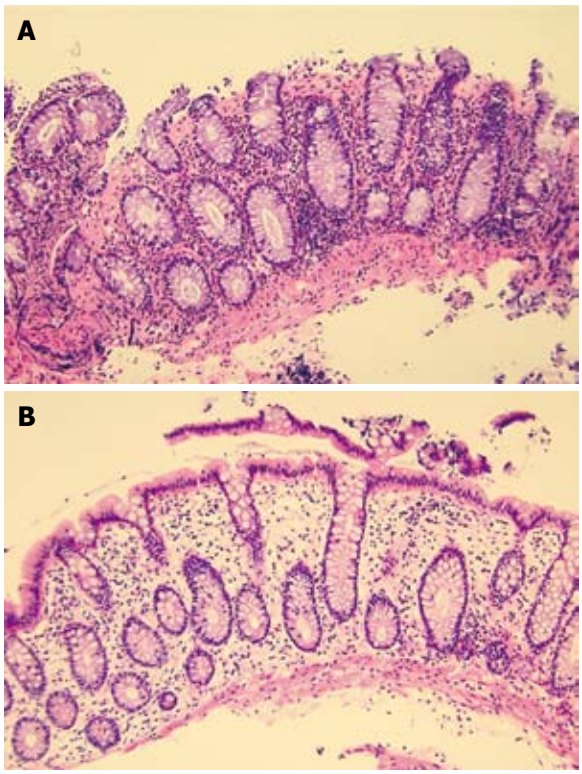

Figure 2 Biopsy specimens taken on April 16 (A) and May 17 (B), 2007 (hematoxylin and eosin staining, × 100). The former showed erosion, moderate infiltration of inflammatory cells in the lamina propria, and subepithelial collagenous thickening. The latter showed disappearance of these abnormalities.

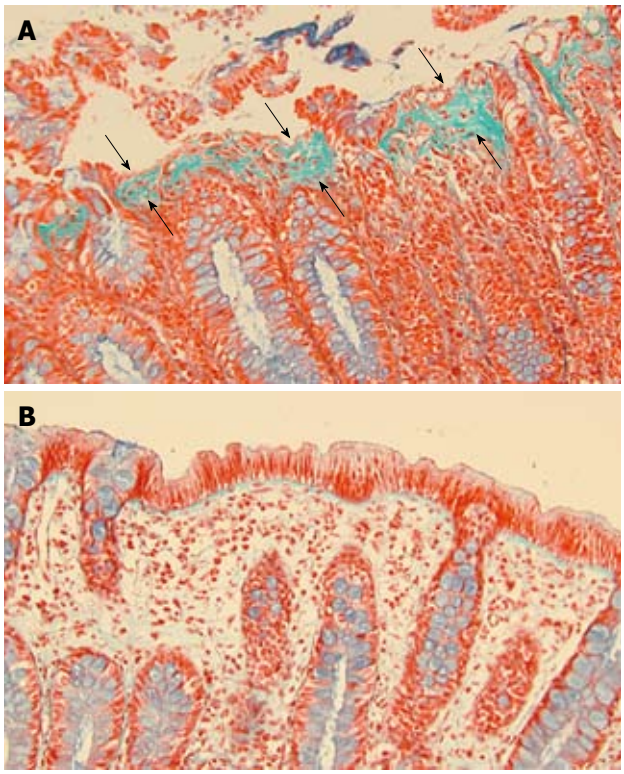

Figure 3 Biopsy specimens taken on April 16 (A) and May 17 (B), 2007 (Masson's trichrome staining, × 200). Subepithelial collagenous thickening (A, arrows) disappeared on May 17 (B).

initiation of lansoprazole to the onset of diarrhea varies extensively from 5 d to 9 mo[9-12]. In our case, it was about 3 and 6 mo in the two episodes, respectively. Treatment of lansoprazole-associated microscopic colitis is withdrawal of lansoprazole. Prompt resolution of diarrhea occurs within 1-10 d[9-12]. In our case, it was 1 d in the first episode and a few days in the second episode. Complete histological normalization subsequently occurred in our case[10,11]. In lansoprazole-associated microscopic colitis, the substitution of omeprazole for lansoprazole was reported to be successful without diarrhea[10].

From the present case it can be concluded that: diffuse cloudiness of colorectal mucosa can be seen endoscopically in collagenous colitis; lansoprazole can cause microscopic colitis; and discontinuation of lansoprazole results in the prompt resolution of diarrhea.

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LETTERS TO THE EDITOR

Emerging clinical and therapeutic applications of *Nigella sativa* in gastroenterology

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Abstract

Nigella sativa (*N. sativa*) decreases DNA damage and thereby prevents initiation of carcinogenesis in colonic tissue secondary to exposure to toxic agents such as azoxymethane. *N. sativa* is of immense therapeutic benefit in diabetic individuals and those with glucose intolerance as it accentuates glucose-induced secretion of insulin besides having a negative impact on glucose absorption from the intestinal mucosa. *N. sativa* administration protects hepatic tissue from deleterious effects of toxic metals such as lead, and attenuates hepatic lipid peroxidation following exposure to chemicals such as carbon tetrachloride.

Key words: *Nigella sativa*; Thymoquinone; Colon cancer; Glutathione-S transferase; Schistosomiasis

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TO THE EDITOR

Yildiz *et al*[1] in their recent article, published in the September issue of the "*World Journal of Gastroenterology*", have clearly highlighted the efficacy of *Nigella sativa* (*N. sativa*) in relieving the deleterious effects of ischemia reperfusion injury in the liver. Their findings bring into highlight the increasing clinical and therapeutic applications of *N. sativa* and its derivatives in the field of gastroenterology.

N. sativa decreases DNA damage and thereby prevents initiation of carcinogenesis in colonic tissue secondary to exposure to toxic agents such as azoxymethane[2]. In fact, sustained delivery of thymoquinone (derived from *N. sativa*) is almost as effective in causing apoptosis of colon cancer cells as sustained delivery of 5-fluorouracil[3]. Similarly, hepatic metastasis from tumors such as mastocytomas is markedly decreased following administration of *N. sativa*[4]. *N. sativa*, when used in combination with *Hemidesmus indicus* and *Smilax glabra*, also seems to decrease hepatic carcinogenesis secondary to exposure to agents such as diethylnitrosamine[5]. These anti-carcinogenic effects are mediated in part by thymoquinone secondary to its inhibitory influence on the NF-κB activation pathway[6].

N. sativa is of great therapeutic benefit in diabetic individuals and those with glucose intolerance, as it accentuates glucose-induced secretion of insulin, besides having a negative impact on glucose absorption from the intestinal mucosa[7,8]. In fact, *N. sativa* attenuates the damage to β-cells of the pancreas following exposure to toxic elements such as cadmium[9]. Similarly, *N. sativa* administration attenuates the ulcerative effects of ethanol on gastric mucosa by decreasing the glutathione-S transferase levels in gastric mucosa[10].

Besides these effects, *N. sativa* also demonstrates anti-parasitic effects. For instance, its administration decreases the number of eggs as well as worms in schistosomiasis, which tends to affect hepatic and intestinal tissues[11]. In addition, *N. sativa* attenuates the side effects associated with some common medications used by gastroenterologists. For instance, cyclosporine, used by gastroenterologists for disorders such as recalcitrant Crohn's disease, is often associated with nephrotoxic side effects, which can be limited by *N. sativa* due to its anti-oxidant properties[12]. Similarly, *N. sativa* administration protects hepatic tissue from deleterious effects of toxic metals such as lead and attenuates hepatic lipid peroxidation following exposure to chemicals such as carbon tetrachloride[13,14].

Thymoquinone, derived from *N. sativa*, has also been demonstrated to induce apoptosis of human colon cancer cells[15]. The above examples clearly illustrate the massive clinical and therapeutic potential of *N. sativa*.

Personally, I believe that the anti-carcinogenic effects of *N. sativa* hold the maximum therapeutic potential. Given the significant benefits associated with its administration, broad-spectrum studies are clearly and urgently needed to further assess and elaborate its therapeutic benefits in gastroenterology.

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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
London, UK
Gastro 2009 UEGW/World Congress of Gastroenterology
www.gastro2009.org

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The major task of *WJG* is to rapidly report the most recent results in basic and clinical research on gastroenterology, hepatology, endoscopy and gastrointestinal surgery fields, specifically including autoimmune, cholestatic and biliary disease, esophageal, gastric and duodenal disorders, cirrhosis and its complications, celiac disease, dyspepsia, gastroesophageal reflux disease, esophageal and stomach cancers, carcinoma of the colon and rectum, gastrointestinal bleeding, gastrointestinal infection, intestinal inflammation, intestinal microflora and immunity, irritable bowel syndrome; liver biology/pathobiology, liver failure, growth and cancer; liver failure/cirrhosis/portal hypertension, liver fibrosis; *Helicobacter pylori*, hepatitis B and C virus, hepatology elsewhere; pancreatic disorders, pancreas and biliary tract disease, pancreatic cancer; transplantation, genetics, epidemiology, microbiology and inflammatory disorders, molecular and cell biology, nutrition; geriatric gastroenterology, pediatric gastroenterology, steatohepatitis and metabolic liver disease; diagnosis and screening, endoscopy, imaging and advanced technology.

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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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insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

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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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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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EDITORIAL

Gastric carcinoids: Between underestimation and overtreatment

Sara Massironi, Valentina Sciola, Matilde Pia Spampatti, Maddalena Peracchi, Dario Conte

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Abstract

Gastric carcinoids (GCs), which originate from gastric enterochromaffin-like (ECL) mucosal cells and account for 2.4% of all carcinoids, are found increasingly in the course of upper gastrointestinal tract endoscopy. Current nosography includes those occurring in chronic conditions with hypergastrinemia, as the type 1 associated with chronic atrophic gastritis, and the type 2 associated with Zollinger-Ellison syndrome in multiple endocrine neoplasia type 1, and type 3, which is unrelated to hypergastrinemia and is frequently malignant, with distant metastases. The optimal clinical approach to GCs remains to be elucidated, depending upon type, size and number of carcinoids. While there is agreement concerning the treatment of type 3 carcinoids, for types 1 and 2, current possibilities include simple surveillance, endoscopic polypectomy, surgical excision, associated or not with surgical antrectomy, or total gastrectomy. Moreover, the recent introduction of somatostatin analogues represents a therapeutic option of possibly outstanding relevance.

Key words: Gastric carcinoids; Endocrine tumors; Well-differentiated tumors; Hypergastrinemia; Chronic atrophic gastritis; Zollinger-Ellison syndrome; Multiple endocrine neoplasia tupe 1; Enterochromaffin-like cells

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INTRODUCTION

The term gastric carcinoid (GC) describes inadequately the pathological continuum of a wide spectrum of distinct neoplasms that arise from gastric enterochromaffin-like (ECL) cells. Carcinoid tumors represent a variety of significantly diverse lesions, which are distinct from adenocarcinomas in their etiology, biological behavior and prognosis. Over the past 5 years, a marked increase in reports addressing GCs has been evident[1]. These tumors are also known by their modern term of gastric neuroendocrine tumors, although the term carcinoid is still commonly used. This review focuses on the biology, diagnosis and treatment of GCs.

EPIDEMIOLOGY

GC tumors that arise from ECL cells have long been considered as rare lesions, and account for less than 2% of all carcinoids tumors and less than 1% of all stomach neoplasms[1-3]. However, recent reviews have indicated that the incidence of GCs may be on the rise[4-6]. In fact, a recent analysis[4] of the National Cancer Institute's (NCI) Surveillance, Epidemiology, and End Results (SEER) database by Modlin *et al* found that, from 1992 to 1999, GCs comprised 8.7% of all gastrointestinal carcinoid tumors. Also, during the period 1950-1999, a total of 562 GCs were recorded in the NCI databases, but from 2000 to 2004, in the SEER database, 1043 new GCs have been reported, which comprises 11.7% of all gastrointestinal carcinoid tumors[7]. On the other hand, a major decline in incidence and mortality of gastric adenocarcinomas has been described over several decades[8]. The male:female ratio for GCs is about 1:2, with 64% of carcinoids found in women, whereas males are almost twice as likely to develop non-carcinoid

Table 1 Characteristics of GC types

	Type 1	Type 2	Type 3
Percentage (%)	70-85	5-10	15-25
Tumor characteristics	Often small, multiple, polypoid, multicentric	Often small, multiple, polypoid, multicentric	Single, > 1-2 cm, polypoid and often ulcerated
Mean age at diagnosis (yr)	63	50	55
Gender	Females > males	Females = males	Males > females
Associated conditions	Chronic atrophic gastritis type A	ZES/MEN1	Sporadic
Serum gastrin levels	Increased	Increased	Normal
pH of gastric juice	Increased	Low	Normal
Ki-67 (%)	Usually < 2	Usually < 2	Usually > 2
Metastases (%)	2-5	< 10	> 50

gastric-cancer (ratio male:female 1.71)[3].

The reasons for the recent marked increase in GCs are unknown, although the wide use of screening upper endoscopy, the routine habit to obtain biopsies in the course of upper gastrointestinal endoscopy, the application of specific immunohistological identification techniques, and a greater clinical focus on the subject may contribute to increased detection of GCs[9]. On the other hand, our knowledge on the biological basis of these tumors, as well as on the complex interplay between genetic and environmental factors that ultimately results in GC development, are still partial. Hypergastrinemia represents a necessary condition for the development of type 1 and type 2 GCs, even if not sufficient[5,10]. The widespread use of proton pump inhibitors can also induce gastric achlorhydria, thus contributing to hypergastrinemia[11,12], even if it is not clear that it has a real association with an increased risk of GCs. On the other hand, the importance of genetic and molecular background remains to be elucidated. Loss of heterozygosity at the multiple endocrine neoplasia type 1 (MEN-1) gene locus 11q13 has been found in all type 2 tumors that are associated with Zollinger-Ellison syndrome/MEN-1, but also in 17%-73% of type 1, and in 25%-50% of type 3 GCs, although these tumors do no develop in MEN-1 patients[13]. A role for the apoptosis-inhibiting protein BCL-2 has also been proposed, with the hypothesis that the anti-apoptotic activity of BCL-2 may contribute to the development of carcinoid tumors by extending the exposure of hyperplastic ECL cells to other so-far-unknown oncogenic factors[14]. Mcl-1 protein expression also increased specifically in human hypergastrinemia-associated type 1 GC tumors. Gastrin-induced mcl-1 expression may therefore be an important mechanism that contributes toward type 1 GC development[15].

CLASSIFICATION

GCs are endocrine tumors of the gastric mucosa that originate from ECL cells[12,13,16-20]. These tumors are classified into three distinct types (Table 1).

Type 1 (GC-1) includes the vast majority (70%-85%) of GCs and is closely linked to chronic atrophic gastritis type A, characterized by decrease acidity, resultant hypergastrinemia and subsequent ECL cell hyperplasia. The spectrum of ECL cell lesions includes hyperplasia (simple, linear and micronodular), dysplasia, and eventually, carcinoids[21]. The lesions are located in the gastric fundus and body and are multicentric, polypoid, small, limited to the mucosa or submucosa, without angioinvasion, well-differentiated, and tend to display benign behavior. It is more frequent in females.

Type 2 (GC-2) accounts for 5%-10% of GCs, is associated with ZES and occurs almost exclusively in the context of MEN-1. MEN-1/ZES patients usually have small duodenal or pancreatic gastrinomas causing hypergastrinemia and subsequent ECL proliferation. The increased incidence of GC-2 in patients with MEN-1 (13%-37%), who display loss of heterozygosity at the MEN-1 gene locus, versus patients with sporadic ZES (0%-2%), supports the genetic role in the pathogenesis of GCs. Type 2 GCs are usually multiple and small, and have low-grade malignancy, although up to 35% of cases are metastatic at presentation. Unlike GC-1, GC-2 is equally frequent in male and female patients[22,23].

Type 1 and type 2 GCs are both associated with hypergastrinemia. In the first case, hypergastrinemia is secondary to hypo/achlorhydria caused by the destruction of gastric parietal cells. In the second case, it is caused by the presence of a primary gastrinoma that, on the contrary, causes hyperchlorhydria. Therefore pH of gastric juice and blood test are useful to discriminate the presence of pernicious anemia by ZES/MEN1. Pernicious anemia is characterized by increased gastric juice pH, low vitamin B12 and presence anti-parietal cells and/or anti intrinsic factor antibodies. The presence of ZES/MEN1 is characterized by low gastric juice pH or better by a basal acid output $\geqslant$ 15 mEq/h. This condition can be investigated by testing a full evaluation of pituitary and parathyroid function, in addition to genetic analysis.

Type 3 (GC-3) represents 15%-25% of GCs, is not related to hypergastrinemia, is characterized by a far more aggressive course, and presents with lymph node and distant metastases in more than 50% of cases. Lesions are typically solitary, larger than 1-2 cm, ulcerated and deeply invasive. They are usually located in the gastric fundus and body, but may occur also in the antrum. This type of GC is more frequent in males[1,3,12,17,18]. Unlike GC-1 and GC-2, GC-3 may be associated with an atypical carcinoid syndrome that

Table 2 Clinicopathological characteristics of endocrine tumors of the stomach according to WHO classification[23]

Well-differentiated tumor-carcinoid
Benign behavior: confined to mucosa-submucosa, non-angioinvasive, ≤ 1 cm in size, non-functioning
ECL cell tumor of corpus-fundus associated with hypergastrinemia and chronic atrophic gastritits (CAG) or MEN1 syndrome
Serotonin-producing tumor
Gastrin-producing tumor
Uncertain behavior: confined to mucosa-submucosa, > 1 cm in size, or angioinvasive
ECL cell tumor with CAG or MEN1 syndrome or sporadic
Serotonin-producing tumor
Gastrin-producing tumor
Well-differentiated endocrine carcinoma-malignant carcinoid
Low-grade malignant, deeply invasive (muscularis propria or beyond), or with metastasis
Nonfunctioning
ECL cell carcinoid, usually sporadic, rarely in CAG or MEN1 syndrome
Serotonin-producing tumor
Gastrin-producing tumor
Functioning
ECL cell carcinoid with atypical carcinoid syndrome
Serotonin-producing carcinoid with syndrome
Gastrin-producing carcinoma-malignant gastrinoma
ACTH-producing carcinoma with Cushing syndrome
Poorly differentiate endocrine carcinoma-small cell carcinoma, high grade malignant, usually non-functioning, occasionally with Cushing syndrome

Table 3 Proposed TNM staging system for GC tumors[7]

	Primary tumor		
	Depth of invasion		Size
T1	Up to and including muscularis propria		≤ 3 cm
T2	Beyond muscularis propria		≤ 3 cm
T3	Up to and including muscularis propria		> 3 cm
	Beyond muscularis propria		> 3 cm
	Lymph node		
N0	No lymph node metastasis		
N1	Regional lymph node metastasis		
	Distant metastasis		
M0	No distant metastasis		
M1	Distant metastasis		
Disease stage	T	N	M
Ⅰ	T1	Any N	M0
Ⅱ	T2	N0	M0
	T3	N0	M0
Ⅲ	T2	N1	M0
Ⅳ	T3	N1	M0
	Any T	Any N	M1

presents with itching, bronchospasm and cutaneous flushing, thought to be mediated by histamine released from ECL cells[1].

Also, type 4 GCs (GC-4) have been described[17]. This type of tumor is not derived from ECL cells, but from other endocrine cells of the stomach, such as those producing serotonin or gastrin. These tumors may have a very aggressive course and may be located in the gastric fundus, body or antrum.

According to the WHO classification[24], type 1 GCs are well-differentiated endocrine tumors with a benign or, more rarely, an uncertain behavior. Type 2 GCs are usually well-differentiated endocrine tumors, but may also be well-differentiated endocrine carcinomas with angioinvasion, invasion of muscularis propria, and metastases at regional lymph nodes, or less frequently at distant sites. Also, occasionally poorly differentiated endocrine carcinomas have been found in patients with ZES/MEN-1. Type 3 CGs may be well-differentiated endocrine tumors or carcinomas, but usually are poorly differentiated endocrine carcinomas with high mitosis rates and Ki-67 values, and regional and distant metastases (Table 2). Moreover, recently, a tumor-node-metastasis (TNM) staging, and a grading system, based on the proliferative status (mitotic count and Ki-67 index) have been suggested for GCs[7] (Table 3), but remain to be validated in clinical practice.

DIAGNOSIS

Diagnosis is currently made during upper gastrointestinal endoscopy performed for a variety of clinical reasons, such as abdominal pain, gastrointestinal bleeding, anemia and dyspepsia. The diagnostic accuracy and the correct characterization of GCs necessitate extensive sampling from both the antrum (two samples) and body-fundus (four samples), in addition to biopsies/removal of the largest polyps. Proliferation rate and degree of dysplasia of gastric endocrine cells may often be difficult to identify with standard histopathological procedures. Histochemistry with chromogranin A (CgA) and synaptophysin assessment is of relevance in identifying hyperplasia, dysplasia and malignant transformation of ECL cells[20,25,26]. Also, immunohistochemical determination of the proliferative index Ki-67 and evaluation of the mitotic index, by counting number of mitosis per 10 high-power fields, are mandatory[27], with a negative prognostic meaning when Ki-67 is > 2% and mitotic index is > 2.

Endoscopy and sampling for histology are currently

considered sufficient when faced with small type 1 and type 2 GCs, reserving endoscopic ultrasound (EUS) for tumors > 1 cm in size[27]. EUS can give information about the location and depth of lesions and local spread, or even highlight the primary gastrinoma in GC-2. EUS can also allow fine-needle aspiration of submucosal lesions.

Computed tomography, magnetic resonance imaging and somatostatin receptor scintigraphy are required for larger tumors, those shown to be invasive by EUS, and type 3 GC, in order to detect distant metastases[27]. The minimal biochemical tests in GC patients include serum gastrin and CgA levels, the most important generic marker for neuroendocrine tumors, with evaluation of gastric juice pH. These tests should be performed at diagnosis. Moreover, determination of CgA could be of relevance in the course of follow-up[5,21,27].

PROGNOSIS

GCs are usually considered as largely benign in prognosis, even if it depends on the type of GC tumor and the extent of the disease. Prognosis ranges from an indolent course for type 1 GCs to the worst one for type 3 GCs.

Rappel and colleagues[28] reported an overall survival rate of 78% in 110 patients with GCs, with the highest rate (100%), when aged-corrected, in the 88 patients with GC-1. Therefore, the authors concluded that patients with GC-1 tumors have a life expectancy comparable to that of the general population. Type 2 GCs have a similar outcome to type 1 GCs, although their overall survival is closely related to the course of the associated gastrinoma, with a 5-year survival of 62%-75%[29]. Type 3 GCs have the worst prognosis and are typically associated with an overall 5-year survival of < 50%[2]. On the other hand, in an update of the SEER database study by Modlin *et al*[4], the 5-year survival rate was 63% for all GCs, 21.2% for metastatic disease, and only 69.1% in the subset of patients with localized lesions. Moreover, a cumulative analysis of GCs in the SEER database from 1992 to 1999 has indicated that distant metastases or regional spread were evident in 10%-30% of cases at the time of diagnosis, thus suggesting that the widespread opinion regarding the benign behavior of GC tumors should be revised.

A further frustrating finding is represented by the lack in the last 30 years of changes in mean overall survival for patients with GCs, as well as for those with other gastroenteropancreatic neuroendocrine tumors[9,30], despite the increased proportion of patients diagnosed at an earlier stage of the disease. However, it should be noted that many variables, other than types of GC, can affect the overall prognosis, such as age, gender, ethnicity, tumor size, depth of invasion, lymph node involvement, distant metastasis, degree of differentiation, and histological subtype.

MANAGEMENT

The clinical approach to GCs is largely dependent upon the type and size of lesions (Figure 1). Management of type 3 GC is fairly clear and comparable to that used for gastric adenocarcinomas, which includes partial or total gastrectomy with extended lymph node resection[1,3,12,16] in the absence of visceral metastases, or systemic chemotherapy if surgery is not feasible, even if, so far, the results are not very encouraging. The questionable efficacy of conventional cytotoxic chemotherapy has prompted investigation of novel therapeutic approaches for patients with advanced carcinoid. These include the use of targeted radiotherapy, as well as regimens incorporating inhibitors of angiogenesis (e.g. bevacizumab) and small molecule tyrosine kinase inhibitors (e.g. sunitinib). The treatment of metastatic liver disease includes hepatic resection, embolization of the hepatic artery, radiofrequency ablation and cryoablation[31].

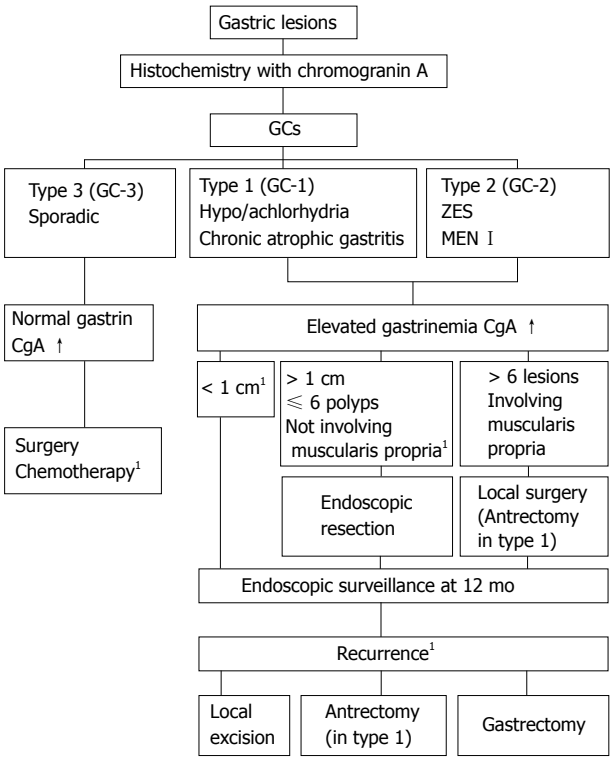

Figure 1 Management flow chart of GCs according to ENETS guidelines[26]. [1]Consider SSAs.

We consider the more controversial management of types 1 and 2 GCs, which are characterized by more benign biological behavior. In GC-1, a conservative approach based on endoscopic resection seems to be the treatment of choice when the size (< 1 cm) and the number (< 3-5) of the tumors render it feasible[1]. However, recently the European Neuroendocrine Tumor Society (ENETS) Consensus Guidelines[27] have suggested that annual surveillance is appropriate when dealing with patients with type 1 GC of less than 10 mm in size. This practical approach is supported by some reports[28,32,33] that suggest that the above careful endoscopic follow-up represents a reasonable and safe option in selected patients. However, further studies including a more consistent number of patients and

with an adequately long follow-up are necessary to support this statement. In fact, despite their usually benign biological and clinical course, type 1 GCs can sometimes exhibit a not entirely negligible mortality rate, as deducible from series with long follow-up[4]. In case of tumors > 10 mm and with up to six polyps not involving the muscularis propria at EUS examination, endoscopic resection remains the reference approach[27]. In the presence of deep gastric parietal wall invasion and positive margins following endoscopic mucosal resection, surgical resection of the tumor should be carried out[27]. Once again, it should be noted that, with these tumors often being multiple and recurrent, antral resection, aimed at avoiding chronic ECL cell stimulation by ongoing hypergastrinemia, is recommended, which is effective in 80% of type 1 tumors[27,34-36]. Moreover, in the case of malignant transformation or recurrence despite local surgical resection, partial or total gastrectomy with lymph node dissection should be performed, as suggested by current guidelines[27].

Overall, despite a generally benign prognosis, the recommended approach in selected subgroups of GC-1 patients appears disproportionately aggressive, and the long-term benefits of antrectomy are still uncertain[34]. Indeed, in some cases, the tumors may become autonomous and no longer gastrin-dependent, and therefore, continue to grow after antrectomy. An octreotide suppression test has been proposed[37] to predict the beneficial outcome from antrectomy, by measuring histidine decarboxylase (HDC) mRNA in the pre- and post-treatment biopsy specimens. In fact, HDC is the enzyme that catalyzes the synthesis of histamine from histidine in ECL cells, a process that is gastrin dependent. A marked decrease in HDC mRNA after octreotide administration indicates that the tumor is still likely to be gastrin dependent.

In extreme situations, i.e., when the biological behavior of the tumor is well defined and definitely benign or malignant, the current guidelines are clear and unambiguous. Conversely, they are less clear for GCs with uncertain behavior, which show atypical characteristics, such as elevated Ki-67, or submucosal invasion, even if they are smaller than 1 cm. Moreover in this situation, according to current guidelines, only endoscopic follow-up is indicated, therefore, information about deep invasion and margin infiltration is not available. At present, relevant controversies and doubts remain in these particular subgroups of patients. It should be stressed that the overall approach is based mainly on the tumor size, but this parameter may not represent the only prognostic factor. Recent studies[38,39] have suggested that proliferation indexes such as Ki-67 are of relevance, but the current best aggregate indicators of prognosis and malignancy seem to be the evidence of invasive growth and the presence of regional or distant metastases (TNM staging system)[38]. At present, however, the criteria to delineate the degree of malignancy remain unclear, and the histological analysis often fails to define precisely the likelihood of aggressive or metastatic potential.

Over the last few years, somatostatin analogues (SSAs) have been used in the treatment of patients with either GC-1 or GC-2[40-45], based on their capability to inhibit gastrin release from the antral G cells, thus reducing ECL cell hyperplasia. However, biotherapy is not currently recommended in patients with type 1 and 2 tumors, except in the rare patients with functioning tumors, and in type 2 patients if indicated for an underlying disease (i.e., other endocrine tumors). Preliminary reports[41] have shown that SSAs have some beneficial effects, for example, by reducing the size and number of carcinoids tumors after 6 mo of treatment. Moreover, the treatment with long-acting SSAs given at monthly intervals for a period of at least 6 mo produces significant suppression in gastrin and CgA levels[40]. Overall, however, the best schedule of treatment remains to be defined.

The management of type 2 GC has to be approached in the context of the MEN-1 syndrome that is present in these patients. As for type 1 GC, endoscopic treatment can be an option, whereas gastric surgery should be performed only in highly selected patients, particularly if the histological examination shows the features of poorly differentiated endocrine tumors. The treatment of type 2 GCs is further complicated by the controversies regarding the treatment of gastrinoma in MEN-1. Currently, no definitive evidence exists that surgery decreases the mortality in MEN-1 or the likelihood that clinically important metastases will develop. Then, the question of whether or not to recommend duodenal-pancreatic surgery in patients with MEN-1 who have pharmacologically controllable ZES and no other clinically evident hormonal excess syndrome is a difficult one. In these cases, the SSA octreotide has been demonstrated to be effective at reducing tumor growth[43].

CONCLUSION

A lot of controversies still exist about the optimal treatment of GC tumors. In fact, endoscopic follow-up could have some risk and is expensive, which leads to further examinations. On the other hand, a more aggressive approach, based on endoscopic or surgical resection may represent over-treatment, with possible unnecessary side effects and high costs. Treatment with long-acting SSAs may therefore represent an alternative option that, even if expensive, seems to be both efficient and safe. Based on the current lack of validated recommendations[40,41,44,45], SSAs should probably be reserved for tumors with atypical characteristics or for multiple small tumors, when surgery is not feasible or judged excessive, and when iterative endoscopic removal is too fastidious or impractical.

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EDITORIAL

Signal transduction pathways in liver and the influence of hepatitis C virus infection on their activities

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Abstract

In liver, the most intensively studied transmembrane and intracellular signal transduction pathways are the Janus kinase signal transduction pathway, the mitogen-activated protein kinases signal transduction pathway, the transforming growth factor β signal transduction pathway, the tumor necrosis factor α signal transduction pathway and the recently discovered sphingolipid signal transduction pathway. All of them are activated by many different cytokines and growth factors. They regulate specific cell mechanisms such as hepatocytes proliferation, growth, differentiation, adhesion, apoptosis, and synthesis and degradation of the extracellular matrix. The replication cycle of hepatitis C virus (HCV) is intracellular and requires signal transduction to the nucleus to regulate transcription of its genes. Moreover, HCV itself, by its structural and non-structural proteins, could influence the activity of the second signal messengers. Thus, the inhibition of the transmembrane and intracellular signal transduction pathways could be a new therapeutic target in chronic hepatitis C treatment.

Key words: Liver; Hepatitis C virus infection; Signal transduction pathway; Proliferation; Apoptosis

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INTRODUCTION

Hepatitis C virus (HCV) was discovered by Choo *et al*[1]. in 1989. HCV is included in the Flaviviridae family within the distinct genus of *Hepacivirus*[2]. According World Health Organization (WHO) data, there are currently about 170 million HCV-infected persons worldwide, which is approximately 3% of the human population. In Poland, the number of chronic HCV-infected persons is estimated to be 750 000, which is about 1.4 % of the general population[3]. In the natural history of HCV infection, there is an 80% risk of chronic infection, as well as the high possibility of severe complications such as liver cirrhosis or hepatocellular cancer (HCC).

The main target cell for HCV infection is the hepatocyte, however the virus also infects B lymphocytes and affects other immune system components. The HCV replication cycle is intracellular and requires activation of many transmembrane and intracellular signal transduction pathways, which are mainly activated by cytokines such as tumor necrosis factor α (TNF-α), interleukins (IL-4, IL-6, IL-12 or IL-13), interferons, mitogens hepatocyte growth factor (HGF), epidermal growth factor (EGF) or transforming growth factor α (TGF-α) and growth inhibitors (TGF-β and activine).

TRANSMEMBRANE AND INTRACELLULAR SIGNAL TRANSDUCTION PATHWAYS

Janus kinase (JAK) signal transduction pathway

The JAK signal transduction pathway is activated by more than fifty different cytokines and growth factors. This intracellular pathway operates not only in hepatocytes but also in immune, hematopoietic and neural system cells. After extracellular ligand-receptor interaction, receptor multimerization and the activation

Table 1 STATs activation and function

STAT protein	Activating factor	Activation effect
STAT 1	IFN-α/β (type Ⅰ INF) IFN-γ (type Ⅱ INF)	Antiviral response, inflammation and hepatocyte damage development, apoptosis stimulation
STAT 2	INF-α/β i INF-λ	Antiviral response
STAT 3	IL-6 and its family, IL-10, IL-22, EGF, HCV proteins	Participates in antiviral IFN-α effect, direct cytoprotective and anti-inflammatory influence on hepatocytes
STAT 4	IL-12	Probably plays a critical role in hepatocytes damage during hepatic ischemia/reperfusion injury and in Th1 differentiation
STAT 5	Growth factors	Regulates the genes expression essential for hepatocytes metabolism, growth and differentiation
STAT 6	IL-4, IL-12 and IL-13	Participates in Th2 lymphocytes response during viral hepatitis and decreases hepatocytes damage during hepatic ischemia/reperfusion injury

of JAK1, JAK2, JAK3 and tyrosine kinase 2 (Tyk2) is observed. The receptor-kinase complex phosphorylates cytoplasmic SH-2-containing transcription factors: signal transducers and activators of transcription (STAT) 1, 2, 3, 4, 5, 6. Activated STATs present two main functions: signal and transcriptional by forming homo- and heterodimers, which translocate to the nucleus to influence transcription. STATs are specifically inhibited by protein inhibitors of activated STAT (PIAS)[4] and by suppressor of cytokine signaling (SOCS) through negative feedback control (Figure 1A). SOCS proteins include SOCS 1, 2, 3 and cytokine-induced Src homology 2 protein (CIS), which bind to JAK kinase inhibiting its enzymatic activity[5].

STATs perform different, often opposing functions in the liver. STAT1 is mainly activated by IFN type I (IFN-α/β) and IFN type Ⅱ (IFN-γ). Its essential function in liver is the participation in antiviral immune defense, as well as in the development of inflammation and apoptosis. IFN-α/β and IFN-λ are ligands for STAT2, whose major function is antiviral defense. Membrane the IFN-α/β receptor (IFNAR) is a complex of two subunits: IFNAR1 and IFNAR2. IFNAR2 presents three diverse forms: full-length IFNAR2c is responsible for signal transduction and transcription process, whereas short form IFNAR2b and soluble form IFNAR2a inhibit these processes[6]. The complex IFN-α/β - IFNAR activates JAK1 and Tyk2 kinases. IFN-γ takes effect by IFN-γ receptor (IFNGR): IFNGR1 and IFNGR2. STAT3 function is especially regulated by IL-6 and its family members such as cardiotrophin-1 (CT-1), oncostatin M (OSM), IL-11, leukemia inhibitory factor (LIF) or ciliary neurotrophic factor (CNTF), by IL-10, IL-22, EGF and HCV proteins. STAT3 participates in the acute phase response, stimulates hepatocytes regeneration and regulates lipid and carbohydrate metabolism in the liver[7]. Moreover STAT3 is one of the main anti-HCV-defense elements that acts by increasing the IFN-α antiviral effect and by its direct cytoprotective and anti-inflammatory influence on hepatocytes[8]. IL-6 and its related cytokines bind gp130 receptor protein, which plays a key role in liver regeneration.

Furthermore, Li *et al*[9] confirmed that gp130 activity is independent of the activities of other kinases, such as MAKP. The ligand-gp130 complex activates JAK1, JAK2 and Tyk2. Recently, the influence of HCV infection on STAT1-3 factors was demonstrated. HCV structural proteins C, E2 and non-structural protein NS5A were shown to reduce the number of membrane receptors (IFNAR1 and IFNAR2c) blocking STAT1-3 activation by IFN-α. STAT1-3 are also inactivated by ethanol and increased level of TNF-α, IL-1β and IL-10[7]. As a result, viral replication, as well as inflammation and fibrosis in the liver, is augmented and has a negative effect on IFN-α treatment response among patients with severe liver damage. However, HCV does not affect IFN-γ function, and in consequence, STAT1 activation[10]. Moreover, Sun and Gao showed that IFN-γ produced by NK cells inhibits hepatocytes regeneration during HCV infection[11]. STAT4 is the least known transcription factor. STAT4 has been shown to be activated by IL-12 and to play a critical role in hepatocytes damage during hepatic ischemia/reperfusion injury and in Th1 differentiation[12]. STAT5 is mainly activated by growth factors and regulates the expression of genes encoding cytochrome P450, HGF and insulin growth factor 1 (IGF1), which are essential for hepatocytes metabolism, growth and differentiation. STAT6 is regulated by IL-4, IL-12 and IL-13. These factors participate in Th2 lymphocytes response during viral hepatitis and reduce hepatocytes damage during hepatic ischemia/reperfusion injury. A summary of STATs activation and function are shown in Table 1.

MAPK signal transduction pathway

EGF, HGF and TGF-α bind with membrane receptors having intrinsic tyrosine kinase enzymatic activity. Ligand-receptor complex multimerization and autophosphorylation are then observed. Ras proteins and GTP create a transient complex activating RAF kinases and MAPK kinases (MKK), which can activate MAPK by dual phosphorylation of threonine and tyrosine. Activated MAPK phosphorylates transcription factors such as cAMP response element-binding (CREB) and Ets-related transcription factor 1 (ELK-1). The MAPK signal transduction pathway is evolutionarily one of the oldest signal transduction pathways in eukaryotic cells. It contains three different signal tracts: the extracellular regulated protein kinase (ERK, p42/44 MAPK) tract, the stress activated protein kinase (SAPK, p38 MAPK, p38-RK or p38) tract, and the c-Jun-NH2-terminal kinase (JNK, p64/54 SAPK) tract (Figure 1B). All of

these pathways regulate processes such as cell growth, differentiation, maturation, proliferation and apoptosis. In mammalian cells every single pathway is activated by two MKK: JNK by MKK4 and MKK7, ERK by MKK1 and MKK2, and SAPK by MKK3 and MKK6. This dual role of MKK in the activation of the JNK, ERK and SAPK signal transduction pathways is still unclear[13]. It has been shown that ERK play a key role in the regeneration of the majority of eukaryotic cells. However the role of SAPK, especially in hepatocytes regeneration, is as yet undefined. Physiologically, the activity of JNK in the liver is minimal but increases during liver regeneration, probably associated with high hepatic TNF-α levels[14].

TGF-β signal transduction pathway

TGF-β is cytokine family member that plays a key role in the processes of cell growth, differentiation, adhesion, apoptosis, and synthesis and degradation of the extracellular matrix. In the liver, during HCV infection, TGF-β is responsible for hepatocytes regeneration and fibrosis, and for epithelial cells proliferation and differentiation. TGF-β1 serum concentration in patients with chronic liver diseases, including chronic HCV infection, is higher the more severe the liver failure is, confirming the association between this cytokine and hepatic fibrosis[15]. Concurrently, in patients with chronic hepatitis C, TGF-β1 serum concentration decreases and normalizes after successful antiviral therapy[16].

The TGF-β membrane receptor consists of two subunits having intrinsic serine/threonine kinase enzymatic activity: the type Ⅰ receptor (TβR-Ⅰ) and the type Ⅱ receptor (TβR-Ⅱ). After binding of the ligand to TβR-Ⅱ, TβR-Ⅰ is phosphorylated in the GS domain containing many glycine and serine amino acids. Activated TβR-Ⅰ influences receptor-specific R-Smad proteins (Smads) and common-partner Smad (Co-Smad). SMADs are a class of proteins that modulate the activity of transforming growth factor beta ligands. Newly created complexes translocate to the nucleus and stimulate transcription and apoptosis[17] (Figure 1C). During liver regeneration, elevated TGF-β concentration is observed, though it does not give rise to an increase of hepatocytes apoptosis, which is probably linked with parallel augmentation of the concentrations of Smads: Ski and SnoN, and other antiapoptotic proteins such as Bcl-2 and Bcl-X in hepatocytes[18]. HCV, through the NS5A protein, inhibits TGF-β signal transduction pathway activity. NS5A reacts directly with TβR-Ⅰ using the region between amino acids 148 and 237. As a result, Smads phosphorylation, complex creation and its migration to the nucleus are blocked. In contrast, NS5B protein has no inhibitory effect on TβR-Ⅰ. TGF-β pathway inhibition can be the result not only of HCV infection, but also of other viruses such as hepatitis B virus (HBV), adenoviruses and HPV. This effect can be due to the direct interaction between the X protein and Smad4 (HBV), interaction between the E1 protein and R-Smad (adenoviruses) or through the inhibitory effect of the E7 protein on R-Smad and Co-Smad complex formation in the nucleus (HPV)[19].

TNF-α signal transduction pathway

TNF-α is produced by macrophages, monocytes, mast cells and NK cells. TNF-α is one of the main mediators of the antiviral inflammatory response, which enhances lymphocytes proliferation and differentiation, acute phase proteins production and cell apoptosis. Two essential TNF-α membrane receptors are known: TNF-R1 (CD120a, TNF-55r or p55) and TNF-R2 (CD120b or p75). TNF-R1 plays a key role in the liver due to its presence not only in hepatocytes membrane but also in Kupffer cells and hepatic sinusoidal endothelial cells. TNF-R1 consists of three domains: extracellular, transmembrane and intracellular (known as the death domain (DD). Activated TNF-R1 binds, *via* the DD, to an adaptor protein TNFR-associated protein with death domain (TRADD), which afterwards activates Fas associated death domain (FADD) proteins, TNF-associated factor-2 (TRAF-2) and receptor-interacting protein (RIP). All of these proteins influence different signal transduction pathways. FADD activates caspases 8 and 10 leading to Death-Inducing Signaling Complex (DISC) formation, which regulates apoptosis[20], whereas TRAF-2 and RIP activate two tracts taking part in the anti-apoptotic effect of TNF-α: IκB kinase (IKK) and nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB factor), as well as JNK and ERK from MAPK signal transduction pathway[21] (Figure 1D). NFκB is a transcription factor comprising two subunits: p50 having a molecular weight of 50 kDa and p65 (also known as RelA, v-rel reticuloendotheliosis viral oncogene homolog A or nuclear factor of kappa light polypeptide gene enhancer in B-cells 3) having a molecular weight of 65 kDa. The RelA subunit is mainly responsible for the anti-apoptotic function of NFκB. In the cytoplasm, NFκB, with inhibitor proteins IκBα or IκBβ (IKK), creates the inactive form. TRAF-2/RIP activates IKK, which phosphorylates IκB leading to its subsequent degradation in proteasomes. Activated NFκB translocates to the nucleus where it binds with DNA through a zinc finger motif and stimulates transcription of genes encoding cytokines, acute phase proteins, immunoglobulins and adhesion factors[22]. TNF-α linked with TNF-R1 leads, depending on activated cellular proteins, to cell proliferation or apoptosis. Kato *et al*[23] showed that HCV core protein C and, to a lesser degree, NS4B protein, influence cell proliferation and production of proinflammatory cytokines such as IL-1, IL-2, IL-3, IL-6, IL-8, IL-12, TNF-α and INF-β stimulating three diverse pathways through NFκB, activator protein-1 (AP-1) and serum response element (SRE). AP-1 is a complex of homo- or heterodimers encoded by c-jun and c-fos family genes. Moreover, AP-1 stimulates proliferation dependent on growth factors, oncogenes and inflammatory peptides. SRE regulates the promoters of immediate early (IE) genes such as c-fos and PIP92. MAPK cascade activation phosphorylates Elk-1 factor binding with SRE and serum response factor (SRF)[24]. The thus created complexes affect transcription of genes taking part in cell proliferation.

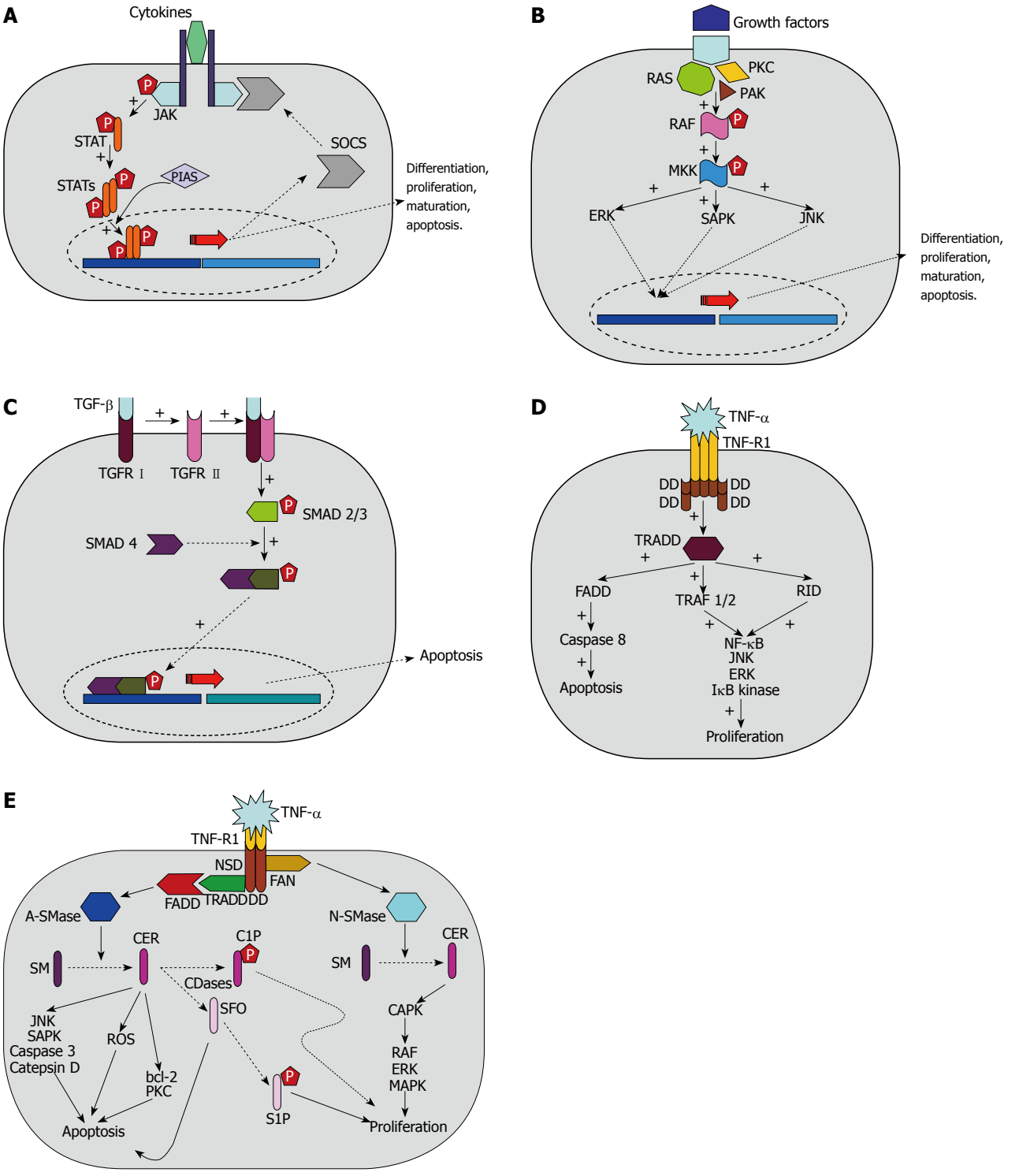

Figure 1 Signal transduction pathway. A: Janus kinase (JAK); B: Mitogen-activated protein kinase (MAPK); C: Transforming growth factor-β (TGF-β); D: Tumor necrosis factor α (TNF-α); E: Sphingolipid. +: Activation; P: Phosphorylation; STAT: Signal transducers and activators of transcription; STATs: Activated STAT; PIAS: Proteins inhibitor of activated STAT; SOCS: Suppressor of cytokine signaling; RAS: Small GTP-binding protein; PKC: Protein kinase C; PAK: P21-activated kinase; RAF: Serine/threonine kinase; MKK: Mitogen-activated protein kinase kinase; ERK: Extracellular signal-regulated protein kinase; SAPK: Stress activated protein kinase; JNK: C-Jun-NH2-terminal kinase; TGFR Ⅰ and Ⅱ: Membrane receptors of TGF-β; SMAD: Class of proteins that modulate the activity of transforming growth factor β ligands; TNF-R1: Membrane receptor of TNF-α; DD: Death domain; TRADD: TNFR associated protein with death domain; FADD: Fas associated death domain; TRAF 1/2: TNF-associated factor-2; RID:Receptor-interacting protein; NFκB: Transcription factor; NSD: TNF-R1 domain activating neutral sphingomyelinase; FAN: TNF-R1 adaptor protein; A-SMase: Acid sphingomyelinase; N-SMase: Neutral sphingomyelinase; SM: Sphingomyelin; CER: Ceramide; C1P: Ceramide-1-phosphate; CDases: Ceramidases; SFO: Sphingosine; S1P: Sphingosine-1-phosphate; ROS: Reactive oxygen species; CAPK: Ceramide-activated kinase.

Sphingolipid signal transduction pathway

Initially sphingolipids were demonstrated to be major components of eukaryotic plasma membranes and mediators of cell-to-cell interactions. Since 1989, many studies have shown that sphingolipids are also the essential second messengers in transmembrane and intracellular signal transduction. This new pathway was called the sphingolipid signal transduction pathway[25]. Generally,

it mediates specific cell reactions such as proliferation, growth arrest, differentiation, apoptosis and calcium homeostasis. It is activated by many proapoptotic and promitotic factors, such as cytokines TNFα and IL-1, Fas (Apo-1, CD95) receptor agonists, CD-40, CD-28, CD-5, DR-5, lymphocyte function-associated antigen-1 (LFA-1), CD-32 (FcγRII), CD-20, hormones (progesterone), vitamin D3, protein kinase C inhibitors, growth factors [platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and nerve growth factor (NGF)], infection (by *P. aeruginosa, S. aureus, N. gonorrhoeae, Sindbis virus* and *Rhinoviru)*, γ radiation, UV and chemotherapeutics (such as doxorubicine and cisplatin)[26]. The final effect of pathway activation (cell survival or death) depends on the inductive factor and the balance between the intracellular levels of its main components: ceramide (Cer) and sphingosine-1-phosphate (S1P). This balance is known as "the Cer/S1P rheostat".

The most intensively studied second messenger of sphingolipid signal transduction pathway is ceramide, which is highly antiproliferative (Figure 1E). Firstly, Cer activates c-Jun kinase (JNK), stress activated protein kinases (SAPK), cathepsin D, methionine adenosyl transferase 1A (MAT1A) and caspase 3, which are responsible for destruction of the cytoskeleton, nuclear and plasma membranes[27]. Secondly, Cer stimulates the mitochondria to release reactive oxygen species (ROS) and cytochrome c, activating the apoptotic proteases[28]. Finally, Cer decreases, by dephosphorylation, the intracellular level of anti-apoptotic proteins of the Bcl-2 family and the activity of anti-apoptotic enzymes like kinases that depend on the intracellular Ca^{2+} levels [protein kinase C, (PKC), PKCα and PKCβa/Akt]. Paradoxically, Cer synthesized from the hydrolysis of sphingomyelin (SM) by neutral sphingomyelinases (NSMases), enhances the activity of the ceramide activated protein kinase (CAPK) and afterwards the serine/threonine kinase Raf and Akt, extracellular signal-regulated protein kinases (ERK 1/2) and the mitogen-activated protein kinase (MAPK). All these kinases stimulate the proliferation process[29]. Cer regulates the cell growth processes through its influence on PKC, kinase suppressor of Ras (KSR), Raf-1, MAPK and ceramide-activated protein phosphatase (CAPP), controlling the protein phosphatases PP1 and PP2. Cer also take a part in plasma membrane reorganization, facilitating transmembrane proapoptotic signal transduction and modulating the autophagocytosis[30]. Autophagocytosis relies on degradation of damaged, dead or used cell structures to prolong cell life. Cer inhibits autophagocytosis by stimulating apoptosis[31].

A further second messenger of sphingolipid signal transduction pathway is sphingosine (SFO). SFO is synthesized from the hydrolysis of Cer by ceramidases (CDases). SFO has a key role in apoptosis by stimulating ROS production in mitochondria and activation of caspase 3, 7 and 8[32]. Additionally, sphingosine inhibits Akt, resulting in the augmentation of the cellular effects of cytochrome c and caspase 3[33]. Moreover, SFO directly blocks DNA synthesis, methylation and replication. SFO also reduces the activity of protein kinases such as PKC, calmodulin-dependent protein kinase and insulin receptor kinase. The PKC inhibition proceeds in two parallel ways: directly and indirectly by decreasing the level of intracellular diacylglycerol (DAG) and Ca^{2+} ions. The PKC inhibition leads to disturbances of nuclear proteins phosphorylation (RNA polymerase, topoisomerase Ⅱ, histones and matrix proteins[34]). Some studies underline the proliferative character of SFO. It seems that low cellular concentrations of SFO stimulate cell proliferation and DNA synthesis, whereas the high concentrations stimulate apoptosis.

Sphingosine-1-phosphate (S1P), synthesized from SFO, has a potent anti-apoptotic character. An increase in the intracellular level of S1P can activate cell proliferation and its passing from G_1 phase to S phase, augment the general number of cells resting in S phase, shorten the time needed for cell division, enhance survival rate of cells subjected to proapoptotic factors, mobilize calcium ions from intracellular compartments, influence cytoskeletal architecture and the processes of cell migration and adhesion. S1P modulates cell functions in two different ways: as an intracellular messenger and as a ligand of G protein-coupled receptors, known as endothelial differentiation genes (Edg) - Edg-1, -3, -5, -6 and -8[35].

Cer may be phosphorylated by ceramide kinase to ceramide-1-phosphate (C1P), which can be dephosphorylated back to ceramide by C1P phosphatase. Similarly to S1P, C1P promotes cell proliferation[36].

Recently, some studies have shown that the inhibition of sphingolipid metabolism can be a new therapeutic target for HCV infection[37].

CONCLUSION

All phases of HCV replication cycle are intracellular and consequently require signal transduction to the host cell nucleus to regulate transcription of viral genes. Although the pathogenesis of transmembrane and intracellular signal transduction during HCV infection is still unclear, it has been shown that HCV could influence activity of the second signal messengers. This mechanism can regulate specific cell mechanisms such as hepatocytes proliferation, growth, differentiation, adhesion, apoptosis, and synthesis and degradation of the extracellular matrix, leading to severe complications of chronic HCV infection such as liver cirrhosis or hepatocellular cancer. For instance HCV, through the NS5A protein, inhibits the TGF-β, signal transduction pathway activity and through the core protein C and, to a lesser degree, the NS4B protein, influences production of proinflammatory cytokines such as TNF-α. Accordingly, it seems that the inhibition of the activity of the intracellular messengers and pathways could be a new therapeutic target for chronic hepatitis C treatment, leading not only to overall HCV elimination from hepatocytes and from other extrahepatic components, but also to decrease the possibility of developing chronic hepatitis C complications. Moreover, the discovery of the role of the JAK signal transduction pathway as the principal signaling pathway for IFN-α opens new research options for a better understanding of IFN-α resistance. HCV structural proteins C and E2 and non-structural protein NS5A have been shown to

reduce the number of membrane receptors IFNAR1 and IFNAR2c blocking STAT1-3 activation by IFN-α. As a result, viral replication, as well as inflammation and fibrosis in the liver, are augmented and has a negative effect on IFN-α treatment response among patients with severe liver damage. Therefore, a better understanding of these signaling defects might lead to new therapeutic strategies, making IFN-α therapy more effective in a larger percentage of patients with chronic hepatitis C infection.

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TOPIC HIGHLIGHT

Kostas Pantopoulos, Associate Professor, *Series Editor*

Non-invasive assessment of liver fibrosis in chronic liver diseases: Implementation in clinical practice and decisional algorithms

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Abstract

Chronic hepatitis B and C together with alcoholic and non-alcoholic fatty liver diseases represent the major causes of progressive liver disease that can eventually evolve into cirrhosis and its end-stage complications, including decompensation, bleeding and liver cancer. Formation and accumulation of fibrosis in the liver is the common pathway that leads to an evolutive liver disease. Precise definition of liver fibrosis stage is essential for management of the patient in clinical practice since the presence of bridging fibrosis represents a strong indication for antiviral therapy for chronic viral hepatitis, while cirrhosis requires a specific follow-up including screening for esophageal varices and hepatocellular carcinoma. Liver biopsy has always represented the standard of reference for assessment of hepatic fibrosis but it has some limitations being invasive, costly and prone to sampling errors. Recently, blood markers and instrumental methods have been proposed for the non-invasive assessment of liver fibrosis. However, there are still some doubts as to their implementation in clinical practice and a real consensus on how and when to use them is not still available. This is due to an unsatisfactory accuracy for some of them, and to an incomplete validation for others. Some studies suggest that performance of non-invasive methods for liver fibrosis assessment may increase when they are combined. Combination algorithms of non-invasive methods for assessing liver fibrosis may represent a rational and reliable approach to implement non-invasive assessment of liver fibrosis in clinical practice and to reduce rather than abolish liver biopsies.

Key words: Chronic liver diseases; Hepatic fibrosis; Liver biopsy; Non-invasive methods for liver fibrosis assessment; Combination algorithms; Decisional tree

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INTRODUCTION

Chronic liver diseases (CLDs) represent a major cause of morbidity and mortality worldwide. The major etiologies are chronic infection with hepatitis B (HBV) and C (HCV) viruses, and alcoholic and non-alcoholic fatty liver disease. Chronic hepatitis B and C are the leading causes of cirrhosis and of hepatocellular carcinoma (HCC) worldwide. Approximately 400 million people are chronically infected with HBV and 25%-40% of them die of cirrhosis and of its end-stage complications[1]. HBV is the most important carcinogen after tobacco and the incidence of HCC is 300 000 cases per year[2]. Chronic hepatitis C is a major health concern with around 200 million individuals affected worldwide, with a greater prevalence in Western countries[3]. Natural history studies indicate that advanced fibrosis and cirrhosis develop in about 20%-40% of patients with chronic viral hepatitis[4,5]. Alcoholic liver disease (ALD) is one of the leading causes of end-stage CLD. It is well established that only a minority of heavy drinkers, estimated at between 10% and 30%, will ever develop advanced ALD and that the risk increases with cumulative alcohol intake[6,7]. Non-

alcoholic fatty liver disease (NAFLD) has become the most common cause of chronic liver disease and impaired liver function in industrialized countries, where 10%-23% of the adult population is estimated to be affected[8,9]. The disease has a spectrum ranging from fatty liver alone to non-alcoholic steatohepatitis (NASH), and progressive steatofibrosis. Many cases of cryptogenic cirrhosis may be end-stage forms of NASH[10]. Hepatic steatosis is currently considered a manifestation of metabolic syndrome[11,12], which is defined as an association of at least 3 of the following disturbances: insulin resistance, central obesity, arterial hypertension, and dyslipidemia, whether hypertriglyceridemia or low HDL-cholesterol levels. Only a percentage of individuals with liver steatosis progress to more advanced stages of the disease[8-10]. The pathogenesis of NAFLD and the reasons why some patients with fatty liver develop NASH and have progressive liver disease are not entirely understood. The most widely supported theory implicates insulin resistance as the key mechanism in NAFLD, leading to hepatic steatosis, and perhaps also to NASH. Obesity, type 2 diabetes, hyperlipidemia and other conditions associated with insulin resistance are generally present in patients with NAFLD[11,12]. A "two-hit" hypothesis has been proposed, involving the accumulation of fat in the liver ("first hit"), together with a "second hit" that produces oxidative stress. Hepatic steatosis has been recognised as the first of two hits in the pathogenesis of NASH, since the presence of oxidizable fat within the liver is enough to trigger lipid peroxidation[13]. However, many patients with fatty liver do not progress to steatohepatitis. Potential second hits for the evolution towards NASH include all mechanisms contributing to the development of inflammation and fibrosis. The presumed factors initiating second hits are oxidative stress and subsequent lipid peroxidation, proinflammatory cytokines (principally tumour necrosis factor alpha), and hormones derived from adipose tissue (adipocytokines)[12]. The progression of liver disease in CLDs presents with a common histopathological pathway which is the formation and accumulation of fibrosis leading to the development of progressive distortion of the hepatic architecture that is the hallmark of evolution to cirrhosis. Liver fibrosis is the result of chronic injury and it appears to play a direct role in the pathogenesis of hepatocellular dysfunction and portal hypertension[14,15]. Development of fibrosis is a progressive process starting from minimal fibrosis limited to the portal tracts, followed by more extensive fibrosis with septa expanding into the liver parenchyma, which can form bridges between two portal tracts or portal tracts and central veins, eventually ending in complete cirrhotic nodules. In patients with CLDs precise definition of the hepatic fibrosis stage is of paramount importance to evaluate the prognosis and the follow-up of the hepatic disease and to decide the need for antiviral therapy in HBV and HCV chronic infections. In CLDs liver biopsy has always been the gold standard for evaluating presence, type and stage of liver fibrosis and to characterize necroinflammation. This procedure, however, presents some limitations since it is invasive, costly and difficult to standardize. Recently, there has been increasing interest in non-invasive assessment of liver fibrosis by using surrogate markers measurable in the peripheral blood or by using instrumental devices, but some concerns about their large-scale clinical use have been raised, based on their performance and validation. This article aims to review the current status of the literature regarding non-invasive assessment of liver fibrosis in CLDs, considering its limitations and advantages. Finally, decisional algorithms to be applied to the most validated and reliable methods in clinical practice are here proposed.

Table 1 METAVIR and Ishak staging systems for liver fibrosis

Description	METAVIR (F)	Ishak (S)
No fibrosis	0	0
Portal fibrosis without septa	1	1-2
Portal fibrosis with few septa	2	3
Septal fibrosis without cirrhosis	3	4
Cirrhosis	4	5-6

Portal fibrosis is a stellate enlargement of portal tracts without any bridging fibrosis on the biopsy sample. Few septa means at least one fibrous septum on the core biopsy. Theoretically, a fibrous septum is a bridge of connective tissue between two portal tracts, a portal tract and a centrolobular vein, or between two centrolobular veins. Septal fibrosis means that the liver biopsy is crossed by several septa; the transition between F2 and F3 by METAVIR or S3 and S4 by Ishak begins when there are more fibrous septa than portal tracts without septa on the biopsy. Cirrhosis means that liver tissue is mutilated by nodular fibrosis that delineates hepatocytes nodules.

HISTOLOGICAL SYSTEMS TO STAGE LIVER FIBROSIS

Several semiquantitative scoring systems have been proposed to stage fibrosis and to grade necroinflammation in the liver. The Ishak's system is a revised version of the older histological activity index[16,17]. It describes grading and staging as two separate items and liver fibrosis is classified as absent (0), mild (1-2), moderate (3-4) and severe/cirrhosis (5-6). This classification system is mainly applied to hepatitis B and C. The METAVIR scoring system for staging has been frequently used in recent times particularly for chronic hepatitis C (Table 1)[18]. Brunt classification of fibrosis assessment is generally used for NASH and it includes five stages: stage 0, no fibrosis; stage 1, zone 3 perisinusoidal or pericellular fibrosis, focally or extensively present; stage 2, zone 3 perisinusoidal or pericellular fibrosis with focal or extensive periportal fibrosis; stage 3, zone 3 perisinusoidal or pericellular fibrosis and portal fibrosis with focal or extensive bridging fibrosis; stage 4, cirrhosis[19]. All these scoring systems have some limits, being semiquantitative, not linear and prone to intra- and inter-observer variation and to sampling variability.

LIVER BIOPSY: IS IT A GOLD OR A SILVER STANDARD?

Liver biopsy has long been the gold standard for staging of

Table 2 Pros and Cons of liver biopsy in staging of hepatic fibrosis

PROS	CONS
Staging of liver fibrosis	Invasiveness (pain, bleeding)
Grade of necroinflammation	Cost (hospitalization)
Steatosis (common in hepatitis C)	Sampling errors
Iron overload (common in hepatitis C)	Possibly refused by patient, concern of physician
Comorbidities (autoimmunity stigmates)	Static data, no information on fibrogenesis

liver fibrosis in CLDs. Liver biopsy has the advantage of obtaining direct information not only about fibrosis, but also about many useful parameters, such as inflammation, necrosis, steatosis, iron or copper deposits. Furthermore, it allows the identification of suspected or unexpected cofactors and comorbidities. However, biopsy is associated with potential morbidity and mortality and has several limitations (Table 2). A single liver biopsy provides static data but with no information on fibrogenesis and fibrolysis that characterise the dynamic processes related to extracellular matrix (ECM) metabolism. Moreover, many recent studies clearly indicate that liver biopsy is prone to sampling errors and may underestimate the amount of liver fibrosis. Cirrhosis could be missed on a single blind percutaneous liver biopsy in 10%-30% of cases[20,21]. When three different liver samples were analyzed, the percentage of correct diagnoses increased from 80% to 100%[22]. In more recent times, Regev *et al*[23] have shown that samples obtained from the right and left lobes of the liver during laparoscopy give different fibrosis staging in one third of cases, with a concordance rate of more than 90% between two experienced pathologists. Other studies have analyzed agreement/disagreement among pathologists. Although the use of more standardized scoring systems, such as those of the Ishak's, METAVIR's and Brunt's classifications, has improved the inter-observer and intra-observer variability, there are still several factors that may significantly influence the reliability of a liver biopsy. The size of the liver sample is very important, especially if we consider that a hepatic sample of 15 mm length represents 1/50000 of the whole parenchyma. Colloredo *et al*[24] have carefully analyzed the impact of the sample size on a correct staging of liver fibrosis in patients with hepatitis C. By reducing progressively the dimensions of the same liver biopsy, they reported that the smaller was the sample analyzed, the milder was the diagnosis made by the pathologist in relation to the stage of fibrosis. Other studies have reported that the type and the size of needle used are also important. The Tru-Cut needle was found to be superior to the Menghini needle, particularly for the diagnosis of more advanced fibrosis[25]. The use of a thicker needle ameliorates the accuracy of the diagnosis but also implies an increased risk of bleeding and perforation for the patient. Interestingly, Rousselet *et al*[26] reported that the degree of experience of the pathologist, as indicated by longer duration of practice or belonging to an academic setting, may have an outstanding impact on the diagnostic interpretation of liver biopsy, even higher than that determined by the one related to sample size. Another shortcoming of liver biopsy is its cost. A cost-benefit analysis showed that in the US the cost of a liver biopsy is 1032 USD and it could rise to 2745 USD when complications occur[27].

Table 3 Features of an adequate liver biopsy sample

Length (mm)	Portal tracts (n°)	Ref.
15	5	[28,29]
20	11	[30]
25	NA	[31]
Bigger is better	NA	[32]

NA: Not available.

LIVER BIOPSY: CONSENSUS AMONG PATHOLOGISTS?

Pathologists have tried to define the features (including length and number of complete portal tracts) of an adequate liver biopsy sample able to reduce the risk of misclassification of liver fibrosis (Table 3). Some authors would suggest that an adequate liver biopsy sample should contain more than 5 portal tracts and be at least 15 mm in length[28,29]. Other studies reported a higher threshold for optimized accuracy. Guido and Rugge have produced a critical review of the literature concerning the use of liver biopsy in chronic viral hepatitis[30]. They suggest that liver biopsy is very often flawed by unacceptable methodological limits and that a biopsy sample of 20 mm or more containing at least 11 complete portal tracts should be considered reliable for adequate grading and staging. Other authors have recommended even bigger samples, up to 25 mm in length[31]. Scheuer has recently concluded that "bigger is better"[32].

LIVER BIOPSY: CONSENSUS AMONG CLINICIANS?

The pathologist's need for obtaining a liver sample of adequate size is in contrast with the patient's need for a procedure causing limited pain and risks. Liver biopsy may in fact be a risky procedure for some patients, particularly for those with more advanced liver fibrosis. Indeed, one third of patients experience pain at the time of the procedure, and the proportion of 0.3%-0.6% of cases presents with serious adverse events like bleeding and even death in decompensated cirrhosis[33]. A French survey which interviewed 1177 general practitioners concluded that liver biopsy may be refused by up to 59% of patients with hepatitis C and that 22% of the physicians share the same concern for the invasiveness of the procedure[34]. On this topic, a survey assessing the consensus among Italian hepatologists on when and how to take a liver biopsy in chronic hepatitis C showed great divergence in the management of the same subgroup of patients[35]. A nationwide survey about assessment of liver fibrosis in hepatitis C among French hepatologist showed

that liver biopsy was still systematically performed by only 4% of respondents. Guidelines for the clinical use of non-invasive methods for assessment of liver fibrosis were required by 95% of the respondents[36].

THE IDEAL NON-INVASIVE METHOD FOR LIVER FIBROSIS

In view of all the shortcomings regarding liver biopsy, in the last decade clinical investigators have been searching for non-invasive methods for accurate information about liver fibrogenesis activity and fibrosis stage in patients with CLDs. Fibrosis is a structural change in the liver that accompanies chronic injury; fibrogenesis refers to the production of ECM. Fibrogenesis increases in response to injury and is essential to tissue repair. The key step in the pathophysiology of liver fibrogenesis is the balance between ECM deposition and removal. An excess of ECM produced after injury stimulates fibrolysis which is mediated by several specific matrix metalloproteinases (MMPs). The hepatic stellate cells (HSCs) are the major source of ECM[14]. Guidelines and Recommendations indicate that staging of liver fibrosis is the most important parameter for the definition of prognosis and for the subsequent management of the patient with CLD[37,38]. Natural history studies indicate that, if only an insignificant rate of patients without fibrosis will develop cirrhosis in the following 5 years, this percentage goes up to 20% for cases with portal fibrosis and to more than 40% for cases with septal fibrosis[39]. Moreover, the decision whether to start an antiviral therapy in cases of chronic viral hepatitis is highly influenced by the staging of liver fibrosis, since treatments are usually long, costly and cause side effects. Identification of patients with cirrhosis is essential to start screening for end-stage liver complications, including esophageal varices (OV) and HCC. International guidelines have defined two stages of liver fibrosis that significantly modify the management of the patients in clinical practice[37,38]: (1) Significant fibrosis, defined as a liver fibrosis stage (F) $\geqslant$ 2 according to METAVIR for hepatitis C or (S) $\geqslant$ 2 according to Ishak for hepatitis B. Significant fibrosis is a definitive indication to start antiviral therapy in chronic hepatitis B and in chronic hepatitis C due to difficult-to-treat genotypes (HCV-1 and HCV-4). For patients infected with HCV genotype 2 or 3 histological definition is not necessary except for those cases with relative contraindications, not motivated or elderly age. The recent Italian Guidelines on the management of chronic hepatitis B have underlined the importance of the stage of liver fibrosis not only in deciding who to treat, but also in deciding the first choice treatment: interferon for mild-moderate fibrosis and nucleoside/nucleotide analogues for cirrhosis, especially if decompensated[38]. (2) Hepatic cirrhosis, defined as liver fibrosis stage of (F) 4 by METAVIR and of (S) 6 by Ishak. Cirrhosis, even when fully compensated and still clinically occult, requires a different and more specific management than simple chronic hepatitis, including screening for OV with annual gastroscopy and for HCC with ultrasound and alpha-fetoprotein every 6 mo.

Table 4 Features of the ideal non-invasive method for liver fibrosis

Reliable (high diagnostic accuracy)
Widely available (simple, least expensive)
Providing information on both fibrosis stage and fibrogenesis activity
Validated by large-scale studies
Validated by independent studies (different authors from the proposing study)
Validated in various etiologies of CLDs (HCV, HBV, ALD, NAFLD)
Identifying clinically important fibrosis stages (significant fibrosis and cirrhosis)

CLDs: Chronic liver diseases; ALD: Alcoholic liver disease; NAFLD: Non-alcoholic fatty liver disease.

The ideal marker test would be able to accurately stage disease and also be sensitive to changes in fibrosis induced by the natural course of disease progression or by therapy (Table 4). Non-invasive methods for detecting liver fibrosis may be divided in two main groups: markers measured in peripheral blood, which could be single parameters or panels combining more parameters, and a technical device that measures the liver stiffness through transient elastography (fibroscan).

SERUM NON-INVASIVE MARKERS OF LIVER FIBROSIS

Among the proposed markers in the literature, some are directly linked to the modifications in ECM turnover occurring during fibrogenesis, the so-called "direct markers", while others reflect alterations in hepatic function but do not directly reflect ECM metabolism, the so-called "indirect markers"[14,15]. The direct markers of liver fibrosis include several glycoproteins (hyaluronan, laminin, human cartilage glycoprotein 39), the collagens family (procollagen Ⅲ, type Ⅳ collagen), the collagenases and their inhibitors and a number of cytokines connected with the fibrogenetic process (TGF-β1, TNF-α). These markers have a pathophysiologic rationale since they may be an expression of either deposition or removal of ECM, thus giving information on its metabolism. They may potentially be used not only to stage liver fibrosis, but also to assess the speed of liver fibrogenesis with the most relevant prognostic value, and also to estimate and monitor the efficacy of and the response to antifibrotic drugs. A limitation to the clinical use of direct markers of liver fibrosis is that they are not routinely available in all hospital settings. The indirect markers of liver fibrosis are biochemical parameters that are measurable in the peripheral blood. They are an indirect expression of liver damage and have a statistical association with liver fibrosis stage. While direct markers of liver fibrosis reflect the process of fibrogenesis, indirect markers satisfy the request for a simple and easy-to-perform marker. Both direct and indirect markers for liver fibrosis may be single or a combination of parameters (Tables 5 and 6). Most of them have been proposed and validated in chronic hepatitis C. Table 7 describes the accuracy of various

Table 5 Single serum non-invasive markers for liver fibrosis

Direct markers	Indirect markers
Hyaluronic acid	Platelet count
Laminin	AST, ALT
Procollagen Ⅲ	γGT
Type Ⅳ Collagen	γ-globulins
Metalloproteinases	Albumin
Inhibitors of metalloproteinases	Prothrombin time

serum non-invasive markers for liver fibrosis as reported in the literature. The performance of non-invasive markers is usually expressed as sensitivity, specificity, positive and negative predictive values (PPV, NPV), accuracy, and compared area under the receiving operating characteristic curve (AUROC).

Hyaluronic acid has been extensively studied in hepatitis C while few studies are available in other etiologies. Overall, a rather good accuracy of this marker in the different CLDs has been reported for detection of significant fibrosis, with an AUROC ranging from a minimum of 0.82 to a very good 0.92[40-46]. In a study conducted in 326 patients, the AUROC was 0.86 and the specificity was 95% for significant fibrosis while the AUROC was 0.92 and the specificity was 89.4% for cirrhosis when a cut off level of 110 μg/L was used[45]. However, another cohort study with more than 400 cases has reported an AUROC of only 0.73 for significant fibrosis[42]. In the same study, cirrhosis could be excluded with excellent NPV and sensitivity (100%) and with excellent AUROC (0.97) using a cut off level of 50 μg/L. Similar results were reported in another study of 486 patients in which hyaluronic acid levels < 60 μg/L excluded cirrhosis with 99% NPV[40]. In ALD the performance of hyaluronic acid for significant fibrosis varied significantly[43,46] while the marker showed very good performance for cirrhosis, with an AUROC of 0.93[46]. The results of a study conducted in 79 patients with NAFLD were also encouraging, since hyaluronic acid had a 0.92 AUROC value for cirrhosis[44]. On the basis of its good accuracy, especially for exclusion of cirrhosis, hyaluronic acid has also been used in panels combining other serum non-invasive markers for liver fibrosis. Recently it has been proposed in combination with AST-to-platelet ratio index (APRI) in hepatitis B. In this study, a combination of APRI > 1.5 and of hyaluronic acid > 300 ng/mL had 98.9% specificity and 93.7% PPV[47]. Laminin is another component of ECM that has been studied as a non-invasive marker. Serum levels of laminin have been used by several authors as a non-invasive parameter to assess liver fibrosis in ALD patients as well as in those presenting with viral hepatitis and hemochromatosis[48]. This determination, however, was progressively discontinued as it did not demonstrate superiority to those of other components of the ECM such as hyaluronic acid. It showed 77% accuracy for detection of significant fibrosis in hepatitis C in a detailed study on 243 patients with CLD[49]. With regard to NAFLD, however, the use of laminin serum levels could be further investigated since a single report, which investigated liver fibrosis in 30 overweight patients, showed a rather good accuracy (87%)[50]. Among the collagens, type Ⅳ collagen has been investigated as surrogate marker of liver fibrosis. Type Ⅳ collagen has been studied in hepatitis C and a good performance for significant fibrosis has been reported (AUC = 0.83)[51]. Murawaki *et al*[52] have compared the diagnostic performance of type Ⅳ collagen with that of hyaluronic acid in hepatitis C and reported the superiority of the latter marker. The role of type Ⅳ collagen has also been investigated in 112 patients with NAFLD and its performance has been compared with hyaluronic acid[53]. The results showed a better diagnostic accuracy for type Ⅳ collagen (0.828 *vs* 0.797 AUROC, respectively). Metalloproteinases (MMPs) and their inhibitors (tissue inhibitors of metalloproteinases, TIMPs) have also been proposed as surrogate markers of liver fibrosis. Those reported to have some clinical impact include MMP-2 and TIMP-1[54]. Boeker *et al*[54] reported a very high performance of MMP-2 in detecting cirrhosis (0.97 AUROC). Unfortunately, it has been difficult to obtain good standardization of the method for routine clinical use. Some authors proposed panels of direct non-invasive markers with the aim of increasing the accuracy of the single parameters. Fibrometer combines age, platelets, prothrombin index, AST, α-2-macroglobulin, hyaluronan and urea. In a few studies, the AUROC for significant fibrosis has been reported as 0.89 in hepatitis C, raising to an excellent 0.943 in patients with NAFLD[55,56]. Patel *et al*[57] proposed fibrospect which combines hyaluronic acid, TIMP-1 and α-2-macroglobulin. It showed an AUC 0.832 for METAVIR stages F2-F4 fibrosis with PPV and NPV of 74.3% and 75.8%, respectively. Another model, named Hepascore, combines bilirubin, γGT, hyaluronan, α-2-macroglobulin, age, and sex, and showed in hepatitis C and ALD a quite good performance for diagnosis of significant fibrosis, ranging from 0.78 to 0.85, and excellent performance for cirrhosis, ranging from 0.89 to 0.92[58,59]. Unfortunately, for both these combination panels large-scale, independent validation studies are lacking. The European Liver Fibrosis (ELF) study group proposed a panel of markers combining age, hyaluronan, type Ⅲ collagen and TIMP-1. In a cohort study of more than one thousand patients with a variety of CLDs the panel detected moderate or advanced fibrosis (Scheuer stages 3, 4) with a 0.77 to 0.94 AUROC in hepatitis C and ALD, respectively[60]. The panel has also been recently validated in 196 patients with NAFLD, with 0.90 AUROC for detection of severe fibrosis, that could increase to 0.98 when the original panel was combined with simple markers[61]. Similar results in terms of accuracy have been recently obtained in 112 consecutive pediatric patients with NAFLD[62].

AST-to-ALT ratio (AAR) was one of the first non-invasive markers proposed. It is easily available and without any cost but it showed a highly variable performance in the studies conducted on HCV patients: sensitivity was between 31.5% and 81.3%, specificity was between 55.3% and 97% and accuracy ranged from 60%-83.6%[63,64]. Another concern about this test may be that it does not identify significant fibrosis but only cirrhosis. In a prospective study, we have also validated AAR in 110 patients with chronic hepatitis B and we obtained 78.9% accuracy for the diagnosis of cirrhosis[65]. AST-to-platelet ratio in-

Table 6 Combinations of serum parameters for non-invasive diagnosis of liver fibrosis

Marker	Description	Settings in which validation exists	Ref.
AST/ALT	AST to ALT ratio	HCV, HBV	[63-65,71,72]
APRI	AST to platelets ratio index	HCV, HBV, HIV/HCV	[64-67,72,75-80]
Forns' index	Age, BMI, γGT, cholesterol	HCV, HBV, HIV/HCV	[67,69,72,75]
Fibrotest	Age, gender, α-2-macroglobulin, γGT, haptoglobin, apolipoprotein A1, total bilirubin	HCV, HBV, ALD, NAFLD, HIV/HCV	[65,67,72,76-80]
ELF	Age, hyaluronic acid, type Ⅲ procollagen, TIMP1	HCV, ALD, NAFLD	[60-62]
Hepascore	Bilirubin, γGT, hyaluronic acid, α-2-macroglobulin, age, sex	HCV	[58,59]
Lok index	AST, ALT, platelets, INR	HCV	[68]
Fibroindex	AST, platelets, g-globulins	HCV	[71,72]
Fibrometer	Age, AST, platelets, hyaluronan, INR, α-2-macroglobulin, urea	HCV	[55,56]
Fibrospect	α-2-macroglobulin, hyaluronan, TIMP1	HCV	[57]
Fib-4	Age, AST, ALT, platelets	HCV, HBV, HIV/HCV	[73-75]

APRI: AST-to platelet ratio index; ELF: European liver fibrosis study group.

Table 7 Performance of several serum non-invasive markers for liver fibrosis (single or combination) as expressed as AUROC

Serum marker	Significant fibrosis	Cirrhosis	Ref.
Hyaluronic acid	0.73-0.92	0.85-0.97	[40-47]
Laminin	0.82	NA	[48,49]
Type Ⅳ collagen	0.83	NA	[51-53]
MMP-2	0.59	0.97	[54]
TIMP-1	0.71	0.90	[54]
ELF	0.77-0.94	NA	[60-62]
AAR	NA	0.51-0.83	[63-65,71,72]
Forns' index	0.75-0.86	NA	[67,68,70,72]
APRI	0.69-0.88	0.61-0.94	[15,64-67,72,76-80]
Fibrotest	0.74-0.87	0.71-0.87	[15,65,67,72,76-80]
Fibroindex	0.74-0.83	NA	[71,72]
Fibrometer	0.89-0.96	NA	[55,56]
Fibrospect	0.83	NA	[57]
Fib-4	0.79-0.85	0.80-0.91	[73-75]
Hepascore	0.82-0.85	0.90-0.94	[58,59]

AUROC: Area under the receiving operating characteristic curve; MMP-2: Metalloproteinase 2; TIMP-1: Tissue inhibitor of metalloproteinases 1; ELF: European liver fibrosis study group; AAR: AST-to-ALT ratio; APRI: AST-to-platelet ratio index.

dex (APRI) is a simple and cheap ratio between AST and platelets, easily available in the clinical practice. It classifies both significant fibrosis and cirrhosis but around 50% of the cases result as unclassified. APRI performance is variable among the studies on hepatitis C: sensitivity ranges between 41% and 91%, specificity between 47% and 95% and accuracy between 60% and 82.7% for significant fibrosis; for cirrhosis, sensitivity ranges between 38.4% and 65.8%, specificity between 86.7% and 93% and accuracy between 60% and 88.4%[15,66,67]. We have also validated APRI in hepatitis B, obtaining 76.1% accuracy for the diagnosis of significant fibrosis and 79.2% for the diagnosis of cirrhosis[65]. Most recently, APRI has been modified into Lok index by adding alanine aminotrasferase (ALT) and international normalized ratio (INR), with further improvement of the diagnostic accuracy, particularly for cirrhosis[68].

Forns' index is a simple panel resulting from the combination of age, γGT, cholesterol and platelets. It does not give any information about cirrhosis, but only about significant fibrosis. Around half of the cases cannot be classified. In hepatitis C, the accuracy reported in various studies was variable (between 50% and 85%)[67,69]. We have also validated Forns' index in hepatitis B, obtaining 64.8% accuracy for the diagnosis of significant fibrosis[65]. It has been suggested that Forns' index might be less accurate in patients with HCV genotype 3 which is associated with very low cholesterol levels[70]. However, this has not been confirmed by other data[67]. In a study performed on 3690 patients with chronic hepatitis C, a combination panel derived from platelets, AST, and γ-globulin named Fibroindex showed 0.83 AUROC in predicting significant fibrosis[71]. However, following validation studies it showed a lower performance[72]. Another combination of simple markers named Fib-4 was recently proposed and it uses platelets, ALT, AST and age. It showed good performance for detection of severe fibrosis (0.85 AUROC) and even better for the diagnosis of cirrhosis (0.91 AUROC) in chronic hepatitis C[73]. The performance of the panel was also evaluated in a cohort of patients with chronic hepatitis B, with similar accuracy for diagnosis of significant fibrosis (0.81 AUROC)[74]. The validity of Fib-4 as a non-invasive marker for liver fibrosis has also been investigated in patients with HCV/HIV coinfection and the reported accuracy was 0.79 for significant fibrosis and 0.80 for cirrhosis[75]. Fibrotest is a patented test that combines γGT, total bilirubin, haptoglobin, α-2-macroglobulin, apolipoprotein A1, age and gender[76]. To date, it is the most validated non-invasive method for liver fibrosis in various etiologies: HCV, HBV, ALD, NAFLD and HIV/HCV coinfected. Between 2001 and 2008 more than 60 scientific studies have investigated fibrotest and 20 of them are independent with respect to the group that have commercialized the test. Overall, independent studies have investigated fibrotest in more than 3000 patients with CLD, mostly hepatitis C. The accuracy reported ranges from 70%-85%[15,67,76]. Fibrotest has been applied to hepatitis B patients and the accuracy reported varies between 83.3% and 87.3% for significant fibrosis and between 86.1% and 94.4% for the diagnosis of cirrhosis[65,77]. In HIV/HCV coinfected patients AUROC was 0.85 for significant fibrosis and 0.87 for cirrhosis[78]. Fibrotest was also validated in ALD, with excellent results, especially for cirrhosis (0.84

AUROC for significant fibrosis and 0.95 AUROC for cirrhosis)[79]. Fibrotest was also applied in 170 patients with NAFLD and the AUROC for significant fibrosis was 0.86[80]. These results in HIV/HCV coinfected, ALD and NAFLD cases need, however, further confirmation from independent groups. Some conditions may alter the result of fibrotest, including Gilbert syndrome and hemolysis. In these cases the clinician should be cautious in the interpretation of the result and the test should be repeated. Overall, among the various serum markers proposed in the literature, APRI and fibrotest are the most validated in all etiologies, and also validated in many independent studies.

TRANSIENT ELASTOGRAPHY (FIBROSCAN)

Apart from serum markers, another method for non-invasive assessment of liver fibrosis is the measurement of liver stiffness[81]. Transient elastography is measured through a device that is called fibroscan (Echosens, Paris) which is composed of an ultrasound transducer probe mounted on the axis of a vibrator. Vibrations of mild amplitude and low frequency are transmitted by the transducer, inducing an elastic shear wave that propagates through the underlying tissues. Pulse-echo ultrasound acquisition is used to follow the propagation of the shear wave and to measure its velocity, which is directly related to tissue stiffness: the stiffer the tissue, the faster the shear wave propagates. Transient elastography measures liver stiffness in a volume that is approximately a cylinder 1 cm wide and 4 cm long, between 2.5 cm and 6.5 cm below the skin surface. This volume is at least 100 times bigger than a biopsy sample. Fibroscan examination is painless, rapid (less than 5 min) and easy to perform at the bedside or in the outpatient clinic. The examination is performed on a non-fasting patient lying flat on his/her back, with the right arm tucked behind the head. The probe transducer is placed on the skin, between the rib bones at the level of the right lobe of the liver where biopsy would be performed. The operator performs 10 valid acquisitions and then the software of fibroscan calculates the median value. The software itself determines whether each measurement is successful or not. Results are expressed in kilo-Pascals (kPa). Liver stiffness values range from 2.5-75 kPa. The results are immediately available and are operator-independent[82]. The exam can be done after a short learning curve (about 100 examinations). The validity of a fibroscan result should be based on two important parameters: (1) the interquartile range (IQR), which reflects the variability of the validated measures, and should not exceed 30% of the median value; (2) the success rate, that is the percentage of valid measurement, should be at least 60%. Despite the exam being relatively easy to perform, the clinical interpretation of results should always be in the hands of an expert clinician who should have at his disposal all clinical information regarding the patient. The result of the fibroscan is given according to cut-off values expressed in kPa: according to the various studies, presence of significant fibrosis is defined by a cutoff value of 7.1 to 8.7, and cirrhosis is diagnosed by a cutoff value of 12.5 to 14.5[83,84]. In various studies, the accuracy of fibroscan results were similar to that of serum non-invasive markers for the diagnosis of significant fibrosis, sometimes with inadequate figures (< 80%). On the other hand, fibroscan showed excellent performance for the diagnosis of cirrhosis (Table 8)[85]. Liver stiffness measurements can be difficult in obese patients or in those with narrow intercostal space and impossible in patients with ascites[81]. Failure rates range between 2.4% and 9.4% in the different studies[81-83,86]. Factors associated with inter- and intra-observator variability were BMI > 25, high grade hepatic steatosis and mild fibrosis (F0-F1 by METAVIR)[82]. A single report suggested that transaminase flares during chronic HBV infection may alter the result of fibroscan because of high flogosis and recruitment of inflammatory cells into the liver parenchyma[87]. Interestingly, a report suggested that acute viral hepatitis increases liver stiffness measured by fibroscan, thus the authors recommend that the extent of necroinflammatory activity needs to be carefully considered in future studies, particularly in patients with absent or low-stage liver fibrosis[90]. Non-invasive assessment of liver fibrosis with fibroscan has also been applied to ALD with 0.91 AUROC for significant fibrosis and 0.92 for cirrhosis[91]. Table 9 summarizes the main limitations of fibroscan. A recent meta-analysis concluded that for the diagnosis of significant fibrosis, transient elastography cannot be used sufficiently in clinical practice. Inclusion of transient elastography in an algorithm with a combination of non-invasive serum markers may be considered[92]. Transient elastography can be used in clinical practice as an excellent tool for the confirmation of cirrhosis when other clinical signs and examinations are non-decisive.

Table 8 Accuracy of fibroscan for the diagnosis of significant fibrosis and cirrhosis

Ref.	Etiology	Accuracy for ⩾ F2	Accuracy for F4
[81]	HCV	88	99
[83]	HCV	83	95
[84]	HCV	79	95
[86]	HCV	80	96
[87]	HCV	NA	95
[88]	HBV	87	88
[89]	HBV	90	94

COMBINATION ALGORITHMS AND IMPLEMENTATION OF NON-INVASIVE METHODS FOR LIVER FIBROSIS IN CLINICAL PRACTICE

The accuracy of most non-invasive methods for liver fibrosis showed variability among different studies and is still considered inadequate to substitute for liver biopsy and for implementation of non-invasive markers for liver fibrosis in clinical practice[15,29,93]. Some preliminary

Table 9 Limitations of fibroscan in clinical practice

Difficult to perform in obese patients (5% rate failure)
Inter-observer and intra-observer variability influenced by liver steatosis
Influence of ALT flares (HBV reactivation)
Lower performance for diagnosis of significant fibrosis

studies suggested that accuracy of non-invasive methods may improve when they are combined in diagnostic algorithms. We have recently proposed an approach that combines APRI and fibrotest sequentially with the aim of increasing the diagnostic accuracy[67]. This is a rational approach for the use of non-invasive markers for liver fibrosis in clinical practice. Indeed, these markers are used when they present with adequate accuracy, while liver biopsy is used only in those patients in which non-invasive markers showed inadequate accuracy. This approach has been named SAFE (Sequential Algorithms for Fibrosis Evaluation) biopsy and its aim is to reduce the number of liver biopsies that are necessary to correctly stage liver fibrosis and to minimize misclassified cases. Through stepwise modeling, two algorithms were developed with the aim of correctly classifying the two stages of liver fibrosis that are clinically significant: (1) significant fibrosis, (2) cirrhosis. The modeling of the algorithms was aimed at achieving > 90% accuracy and minimizing misclassified cases. In the model APRI has been used as first line test since it is cheap and simple, fibrotest has been used as second line test since it is costly and more complex. Liver biopsy has been used only as third line test in those cases in which the two non-invasive markers did not show adequate accuracy and/or in unclassified cases (only for APRI) (Figures 1 and 2). The modeling of the stepwise algorithms was based on the predictive values of the single markers. In the algorithm for significant fibrosis (Figure 1), 0.5 cut-off of APRI had low NPV to exclude significant fibrosis, while 1.5 cut-off showed high PPV to diagnose significant fibrosis. Similarly, 0.49 cut-off of fibrotest showed high PPV to diagnose significant fibrosis, whereas values less than 0.48 could not accurately exclude significant fibrosis. In the algorithm for cirrhosis (Figure 2), 1 cut-off for APRI showed high NPV to exclude cirrhosis, while 2 cut-off did not show sufficient PPV to diagnose cirrhosis. Similarly, 0.48 and 0.75 cut-offs of fibrotest showed good NPV and PPV, respectively, for cirrhosis, while intermediate values could not give accurate diagnosis.

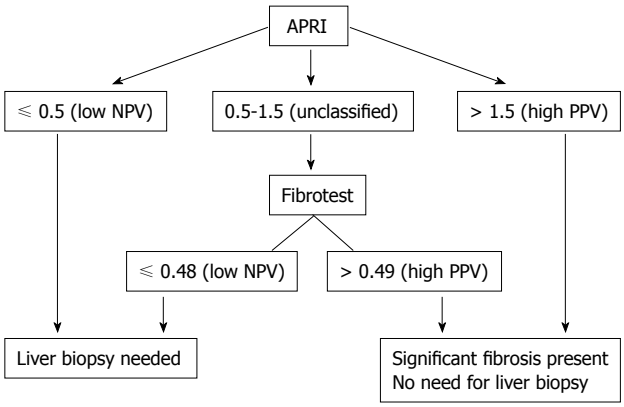

Figure 1 The SAFE-biopsy algorithm for significant fibrosis (≥ F2 by METAVIR). The figure reports the cut-offs used for APRI and Fibrotest in the decisional tree.

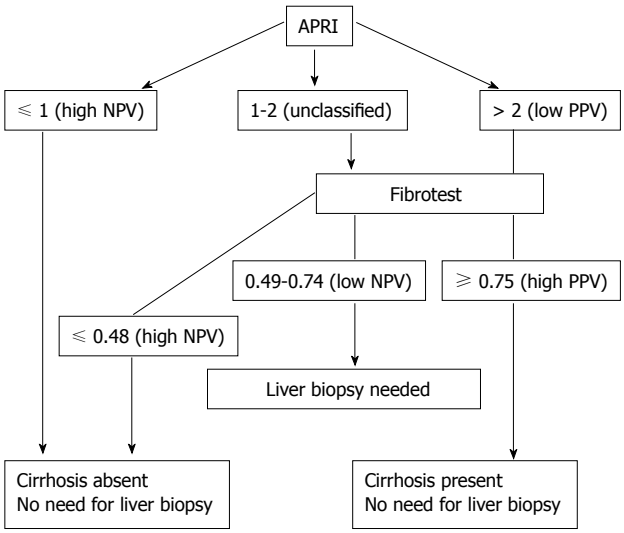

Figure 2 The SAFE-biopsy algorithm for cirrhosis (F4 by METAVIR). The figure reports the cut-offs used for APRI and Fibrotest in the decisional tree.

IMPLEMENTATION OF SAFE BIOPSY IN CLINICAL PRACTICE

In clinical practice, SAFE biopsy can provide the following responses: (1) Presence of significant fibrosis, then indication to administer antiviral therapy; (2) Presence of liver cirrhosis, then indication to specific follow-up with abdominal ultrasound, α-fetoprotein and gastroscopy; (3) absence of cirrhosis; (4) liver biopsy needed to correctly stage hepatic fibrosis.

The main concept of SAFE biopsy is that liver biopsy cannot be completely avoided but can be markedly reduced and limited to those cases in which serum markers for liver fibrosis do not show enough accuracy. Indeed, SAFE biopsy may avoid the diagnostic funnel represented by liver biopsy and it may stimulate general practitioners and patients to perform the initial screening for CLD. With this approach, liver biopsy and non-invasive markers for liver fibrosis are not antagonists, but they are agonists towards the common goal of correctly classifying liver fibrosis. SAFE biopsy has been recently validated in a multicentre, international study on serum non-invasive markers for liver fibrosis. This study, named SAFE protocol protocol, has enrolled more than 2500 cases of patients with CLD in whom APRI and fibrotest were available and liver histology was used as reference standard. The centers involved were from Italy, US, France and Romania. To date, this is the largest independent study on non-invasive methods for liver fibrosis. We have recently presented the results on 2035 cases with hepatitis C and they have confirmed high accuracy and high number of saved liver biopsies[94] (Table 10). The results of an interim analysis conducted on 210 HBV patients also showed high ac-

Table 10 Main features of SAFE biopsy[67,94] for significant fibrosis and cirrhosis in 2035 HCV cases

	Significant fibrosis	Cirrhosis
Sensitivity (%)	100	92.7
Specificity (%)	77	90.4
Accuracy (%)	90	93
AUROC	0.9	0.92
Saved biopsies (%)	47	82

SAFE: Sequential algorithms for fibrosis evaluation; AUROC: Area under the receiving operating characteristic curve.

curacy (> 90%) of SAFE biopsy algorithms for both significant fibrosis and cirrhosis, with a percentage of saved liver biopsies ranging from 45%-82%. We have also compared in 1013 HCV cases the performance of SAFE biopsy with another two algorithms combining non-invasive markers for liver fibrosis that were then proposed: Fibropaca algorithm, based on concordance of Forns' index, APRI and fibrotest; Leroy algorithm, based on concordance of APRI and fibrotest[95-97] (Table 11). Fibropaca algorithm and SAFE biopsy showed a similar accuracy but the latter saved more liver biopsies and allowed us to perform a minor number of non-invasive markers, with a consequent saving in terms of costs. The main advantages of SAFE biopsy include a larger first level screening of liver fibrosis, higher patient compliance and lower screening costs. In some specific settings, SAFE biopsy may show even more efficient results when compared with the diagnostic funnel represented by liver biopsy alone.

ALGORITHMS FOR IMPLEMENTATION IN CLINICAL PRACTICE

Castera *et al*[83] have recently proposed an algorithm which combines fibrotest and fibroscan with the aim of increasing the accuracy of the single non-invasive methods in hepatitis C. This algorithm results in an increased accuracy, especially for the diagnosis of significant fibrosis. A recent collaborative study was aimed at comparing the algorithm combining fibroscan and fibrotest (named Bordeaux algorithm) and SAFE biopsy in 302 patients with hepatitis C[98] (Table 12). The results showed that the Bordeaux algorithm saved more liver biopsies for diagnosis of significant fibrosis, although both algorithms saved a similar number of overall liver biopsies, and Bordeaux algorithm showed a higher overall accuracy for diagnosis of cirrhosis. On the other hand, Bordeaux algorithm uses fibrotest and fibroscan in all patients, while SAFE biopsy uses fibrotest in a subgroup of patients that are not well classified by APRI, which has virtually no cost. The two algorithms could be used for large scale screening of liver fibrosis and the choice of the algorithm may be based on the local availability of the non-invasive methods. Interestingly, the use of either fibroscan or fibrotest has been recently recommended in France by the Haute Autorité de Santé for the first line assessment of liver fibrosis in patients with hepatitis C without comorbidities[85]. Figure 3A and

Table 11 Comparison of the performance of SAFE biopsy[67,94], Fibropaca algorithm[96] and Leroy algorithm[97]. Results are expressed as percentages

	SAFE biopsy for diagnosis of		Fibropaca algorithm for diagnosis of		Leroy algorithm for diagnosis of
	⩾ F2	F4	⩾ F2	F4	⩾ F2
APRI needed	100	100	100	100	100
Forns needed	0	0	100	0	0
Fibrotest needed	41.7	57.6	100	100	100
Sensitivity	100	81.8	85.5	72.7	89.6
Specificity	78.2	92.4	89.9	96.7	97.8
Accuracy	90	91.2	87.6	94	93.5
Saved biopsies	43.8	79.1	51.7	76.2	29.2

⩾ F2: Significant fibrosis; F4: Cirrhosis; APRI: AST-to-platelet ratio index.

Table 12 Comparison of the performance of Bordeaux algorithm[98] and SAFE biopsy[67,94]. Values are expressed as percentages

	Bordeaux algorithm		SAFE biopsy	
	⩾ F2	F4	⩾ F2	F4
APRI needed	0	0	100	100
Fibrotest needed	100	100	43.7	61.9
Fibroscan needed	100	100	0	0
Accuracy	91	93	94	87
Biopsies saved	71.9	78.8	48.3	74.8

B show a rational proposal for the use of non-invasive methods for liver fibrosis in clinical practice, based on the local availability of the different methods and on their performances. A combination approach for clinical use has also been proposed by others[99]. Non-invasive methods for liver fibrosis and combination algorithms may be of paramount importance for the monitoring of progression of liver disease. Indeed, if it is acceptable to perform a liver biopsy at time 0, it is inconceivable however to perform a liver biopsy every year to monitor liver fibrosis progression, while this is feasible with non-invasive methods for liver fibrosis. According to local availability of the methods and attainment of non-invasive markers by the clinician, two different approaches may be used: (1) to fix the value with combined use of biopsy and non-invasive markers at time 0 and then monitoring with non-invasive markers; (2) to use non-invasive markers and then perform a liver biopsy when clinically necessary (Figure 4A and B).

MONITORING OF EFFICACY OF ANTIVIRAL THERAPIES

Apart from the diagnosis of liver fibrosis stage, few recent studies have focused on the possible use of non-invasive methods for liver fibrosis in the monitoring of antiviral therapies. Indeed, especially in hepatitis B, antiviral therapies may be long-term, such as treatments with nucleoside/nucleotide analogues, and the clinician may want to know not only the biochemical or virological response, but also and more appropriately the histological

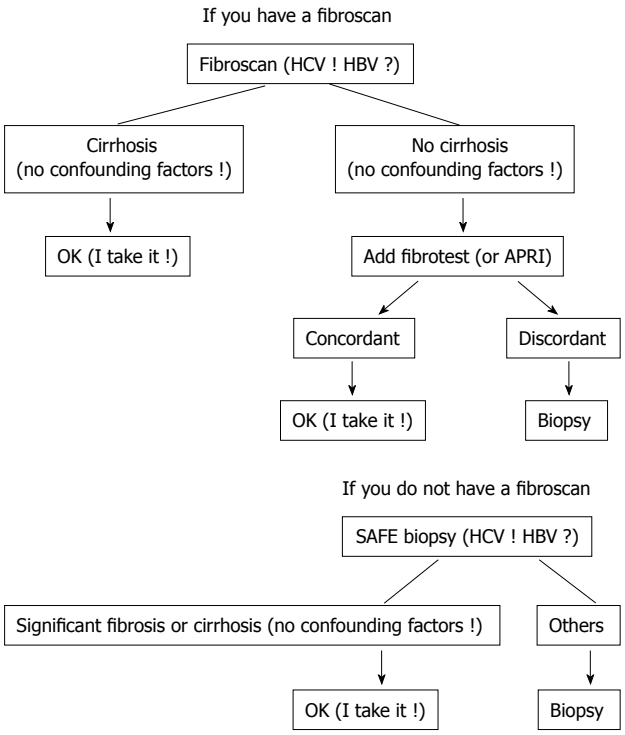

Figure 3 Diagnostic algorithms for implementation of non-invasive methods for liver fibrosis in clinical practice based on the local availability of the most validated methods.

response. Initial reports have shown that both fibrotest and fibroscan values change significantly during and after antiviral therapy in both hepatitis C and B[100-102]. Indeed, a significant improvement in fibrotest and fibroscan value has been reported in patients who achieve sustained virological response (SVR) *vs* those without SVR, and in some cases this was also maintained for 12 mo after therapy[101]. This may mean that there is a regression of liver fibrosis with antiviral treatment but further prospective, large-scale studies are needed.

MONITORING OF LIVER DISEASE COMPLICATIONS

A very attractive application of non-invasive methods for liver fibrosis may be the monitoring of liver disease complications to predict clinical events in compensated cirrhosis. Preliminary results suggest that liver stiffness values in cirrhotic patients may increase as liver disease is more advanced. In a retrospective study of 711 patients with CLD (95 with histologically-proven cirrhosis), liver stiffness values significantly correlated not only with the Child-Pugh score but also with clinical parameters (past history of bleeding varices or ascites, HCC), biochemical parameters (platelets, INR, factor V, albumin and bilirubin) and others (2-3 grade OV, splenomegaly on sonography, nodular surface, heterogeneous parenchyma) of liver disease severity[86]. Cut-off values of 27.5, 37.5, 49.1, 53.7 and 62.7 kPa had > 90% NPV for the presence of grade 2-3 OV, Child-Pugh scores B or C, past history of ascites, HCC and esophageal bleeding,

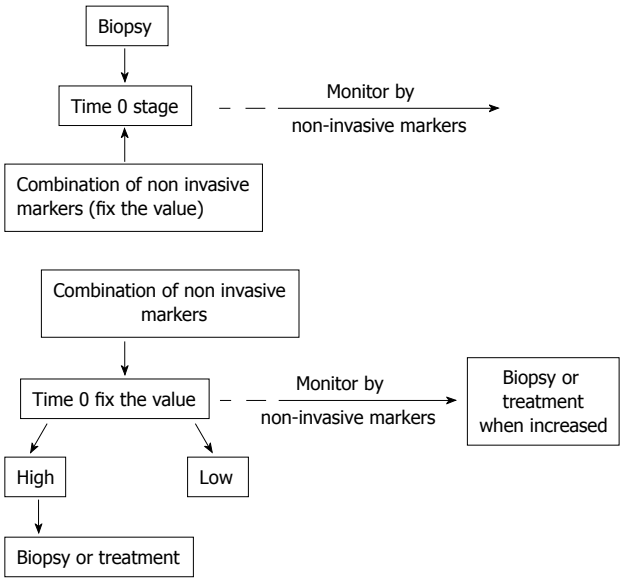

Figure 4 Perspectives for the use of non-invasive markers for long term monitoring of chronic liver diseases.

respectively. More recently, Vizzuti *et al*[103] reported a rather high sensitivity (90%) of fibroscan for prediction of OV with 17.6 kPa cut-off. Other preliminary studies have suggested that some non-invasive markers for liver fibrosis could predict the presence of OV. Sanyal *et al*[104] reported high NPV for excluding grade 2-3 varices when platelets were > 150 000/mm^3. Giannini *et al*[105] reported a good sensitivity (91.5%) with an overall accuracy of 86% for diagnosis of OV with a 909 cut-off of platelet count to spleen diameter ratio. A recent multicenter, international study was aimed at investigating in 510 consecutive cirrhotic patients the role of 7 simple non-invasive markers for liver fibrosis in predicting the presence of OV of any grade and of grade 2-3 OV[106]. The markers analyzed were platelets, AAR, Lok index, APRI, Forns' index, Fib-4, Fibroindex. Presence of grade 2-3 OV could be excluded with > 96% NPV by a specific cut-off of Lok index (1.5). None of the tests were able to predict the presence of grade 2-3 OV due to low PPV. A combination of Lok index (cutoff 0.9) and Forns' index (8.5) could predict the presence of OV of any grade with 88% PPV, 83% accuracy and 0.82 AUC. The conclusion was that, even if simple non-invasive markers for liver fibrosis cannot be a substitute for endoscopy for OV screening, they may be used to stratify cirrhotics by risk. In a recent prospective study of 298 patients with chronic hepatitis C, the performance of fibroscan, fibrotest and simple serum markers for detection of cirrhosis and its complications have been assessed[107]. The authors concluded that fibroscan is the most accurate method for diagnosis of cirrhosis but it cannot replace endoscopy for screening of OV. These preliminary findings are promising but need to be confirmed in long-term prospective follow-up studies.

Several recent studies have reported a correlation between liver stiffness values and portal hypertension,

assessed by measurement of hepatic venous pressure gradient (HVPG) which is considered the gold standard for the diagnosis and staging of portal hypertension[103,108-110]. Carrion *et al*[108] reported a close direct correlation between liver stiffness values and HVPG in 124 HCV-infected liver transplant recipients. More recently, Vizzutti *et al*[103] reported similar results in 61 patients with HCV-related severe CLD (METAVIR F3-F4). Other authors have failed to find similar results[111].

THE FUTURE: GENETICS FOR THE IDENTIFICATION OF PATIENTS WITH CHRONIC LIVER DISEASES AT RISK OF PROGRESSION

The identification of patients at high risk of developing progressive liver disease on the basis of genetic profile may be extremely useful in the future. A recent collaborative study used seven genetic variants to identify patients with hepatitis C at risk for developing cirrhosis, based on the analysis of paired liver biopsies. A cirrhosis risk score (CRS) was calculated on the basis of seven single nucleotide polymorphisms and the patient's gender[112]. In this case, increasing CRS was associated with fibrosis progression in HCV patients presenting with no liver fibrosis. CRS genetic signature could potentially be a useful prognostic indicator of those patients with HCV infection most likely to develop fibrosis progression and/or cirrhosis.

HIGHLIGHTS

Staging of liver fibrosis is essential in clinical practice for the management of patients with CLDs. Nowadays liver biopsy can no longer be considered the exclusive tool for the diagnosis of liver fibrosis since the available data support a rational use of the most validated non-invasive methods for liver fibrosis and especially of their combination algorithms. This is particularly true for chronic hepatitis C, where an adequate validation of some non-invasive methods for liver fibrosis exists. Non-invasive methods for liver fibrosis, when combined, may reduce by 50%-80% the number of liver biopsies needed for correctly classifying hepatic fibrosis. However, liver biopsy cannot be completely avoided but should be used in those cases in which non-invasive methods show poor accuracy. In clinical practice, the choice of the non-invasive method and, especially, of the combination algorithms may depend on their performance and on local availability. Further studies, especially in chronic hepatitis B, ALD and NAFLD, are needed to better assess performance of non-invasive markers in these settings and to develop rational algorithms for implementing non-invasive assessment of liver fibrosis. Future studies should also focus on non-invasive monitoring of antiviral treatment efficacy and cirrhosis complications, and genetic studies for precocious identification of patients who are at high risk of developing end-stage liver diseases.

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REVIEW

Review of salt consumption and stomach cancer risk: Epidemiological and biological evidence

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Abstract

Stomach cancer is still the fourth most common cancer; thus, it remains an important public health burden worldwide, especially in developing countries. The remarkable geographic variations in the rates of stomach cancer indicate that dietary factors, including a range of food groups to which salt and/or nitrates have been added, may affect stomach cancer risk. In this paper, we review the results from ecologic, case-control and cohort studies on the relationship between salt or salted foods and stomach cancer risk. The majority of ecological studies indicated that the average salt intake in each population was closely correlated with gastric cancer mortality. Most case-control studies showed similar results, indicating a moderate to high increase in risk for the highest level of salt or salted food consumption. The overall results from cohort studies are not totally consistent, but are suggestive of a moderate direct association. Since salt intake has been correlated with *Helicobacter pylori* (*H pylori*) infection, it is possible that these two factors may synergize to promote the development of stomach cancer. Additionally, salt may also cause stomach cancer through directly damaging gastric mucus, improving temporary epithelial proliferation and the incidence of endogenous mutations, and inducing hypergastrinemia that leads to eventual parietal cell loss and progression to gastric cancer. Based on the considerable evidence from ecological, case-control and cohort studies worldwide and the mechanistic plausibility, limitation on salt and salted food consumption is a practical strategy for preventing gastric cancer.

Key words: Disease prevention; *Helicobacter pylori* infection; Salt consumption; Stomach cancer

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INTRODUCTION

Stomach cancer is the fourth most common cancer and is the third leading cause of cancer death worldwide[1-3]. The estimated number of stomach cancer cases worldwide was 933 900 in 2002, with two-thirds occurring in developing countries[3]. Tremendous variation in both incidence and mortality rates exists across geographic regions, with > 10-fold differences observed between low-risk and high-risk areas[4]. Although stomach cancer incidence rates have been decreasing slowly over recent decades in China, it was estimated that there were 0.4 million new cases diagnosed and 0.3 million deaths from this malignancy in 2005[5]. Therefore, this disease remains an important public health burden throughout the world, especially in developing counties including China.

Several risk factors for stomach cancer have been identified, including *Helicobacter pylori* (*H pylori*) infection, salt-preserved foods, dietary nitrite, smoking, alcohol, obesity, radiation, and family history[6,7]. Researchers also found that the incidence rates of stomach cancer varied across different geographic regions and this variation may be associated with genetic, lifestyle or environmental

factors, including diet[8]. Salt intake was first reported as a possible risk factor for stomach cancer in 1959[9]. In some early studies, using refrigerators for food storage, which may be an indicator of less salted food consumption or decreased salt intake, was found to be correlated with a reduction in stomach cancer rates[10,11], leading researchers to hypothesize that salt intake may play a role in the development of stomach cancer. A Japanese ecological study suggested a nearly linear correlation between the cumulative mortality rate of stomach cancer and the median 24 h urine salt excretion level[12,13]. Experimental studies[14,15], including rodent models, have also suggested that salt may play an important role in the etiology of stomach cancer. Based on the available experimental and epidemiological data, a report from World Health Organization (WHO)/Food and Agriculture Organization (FAO) Expert Consultation in 2003 concluded that "salt-preserved foods and salt probably increase the risk of stomach cancer"[16].

The purpose of this paper was to review the current literature on salt consumption and the risk of stomach cancer. We obtained the relevant papers and identified our literature search through PubMed from SCI papers. All cohort papers were selected with cohort size more than 2000; case-control papers were filtered out with case sample size around or more than 100. At the end, we summarized the evidence from epidemiological perspectives regarding salt intake and stomach cancer risk.

EPIDEMIOLOGICAL STUDIES OF SALT AND STOMACH CANCER RISK

When evaluating epidemiologic studies on the relationship between salt or salted food consumption and stomach cancer risk, it is essential to consider the diversity of salted foods. Some studies analyzed overall dietary salt intake, whereas others evaluated stomach cancer risk associated with salt intake in various categories, such as table salt or salted fish.

Ecologic studies

Several ecologic studies reported positive associations between different indicators of salt consumption and stomach cancer mortality at the population level[17-21]. In an ecologic study of 24 countries, median urinary sodium levels, ascertained on randomly selected samples from each country, were significantly correlated with stomach cancer mortality (r = 0.70 in men; 0.74 in women) (both $P < 0.001$)[17]. Another study evaluated correlations between both salt intake and 24 h urinary sodium excretion and stomach cancer mortality among men in four geographic regions of Japan, and reported a strong correlation between stomach cancer mortality and salt excretion, but not with dietary salt intake[21]. An ecologic study of stomach cancer mortality in 65 Chinese counties observed significant, yet modest, correlations for intake of salt-preserved vegetables (r = 0.26 in men and 0.36 in women)[18]. A Japanese study administered a 38-item food frequency questionnaire to a sample of 634 men and the wives of 373 of these men from five districts in Japan. The rank correlation coefficient between gastric cancer mortality and pickled vegetable consumption was 0.36[19]. In a similar study of 207 Japanese men and the wives of 165 of the men, average daily sodium consumption, estimated using a 3 d weighed food record, was correlated with stomach cancer mortality rates (partial rank r = 0.45)[20].

In summary, the majority of ecological studies indicated that the average salt intake in each population was closely correlated with gastric cancer mortality. However, employing dietary assessment methods in these studies has some limitations, such as variation of the questionnaire validity in different population, and use of the same composition table calculating salt intake in diverse dietary cultures. One validated questionnaire used by one population may not be appropriate or valid for another population; thus in population based studies, dietary estimates may not be highly accurate and the association between dietary factors and disease may be much compromised. Applying the same composition table to calculate the same food in different dietary culture may cause bias, since the same food in different areas may have different salt content. For example, the regional average salt content of miso in 39 regions of 20 prefectures across Japan ranged from 9.1% to 18.2%[22]. Furthermore, as with all ecologic studies, diet and stomach cancer were neither measured nor analyzed on the individual level. Rather, the diets of sampled individuals were used to represent entire populations or geographic regions. Thus, misclassification is an obvious concern. Furthermore, associations observed at the population level cannot be assumed to hold at the individual level, and causality cannot be inferred from this type of study.

Case-control studies

Forty two case-control studies on salt consumption in relation to stomach cancer are presented in Table 1[23-64]. Of these, twenty two studied overall salt, table salt or sodium[23-44], twelve estimated salted/dried fish and/or salted fish gut or cod roe[25,39,45-54], six investigated salted or pickled vegetables[25,32,52-55], three focused on salty snacks[41,56,57], and twelve studied salted foods in general[29,30,32,52,54,58-64].

Among the sixteen studies that estimated overall dietary salt or sodium intake, eight in Puerto Rico, Spain, Korea, Italy, Mexico, China (two) and USA have shown strong statistically significant increases in risk (OR = 1.5-5.0 for the highest intake levels)[28-31,33,34,36,43]. Seven of them reported statistically non-significant OR of 1.1 to 1.5 for consumption in the upper half of overall salt or sodium intake[24-27,32,35,44], and the remaining study reported no association[23]. Six of the studies specifically examined the use of table salt, with three studies (in Belgium, England, and Poland) reporting statistically significant increases in risk with OR = 1.6, 1.8, 6.2, respectively[38,40,42]. Two other studies each reported statistically non-significant odds ratios of 1.5 for consumption of the upper half of the table salt intake distribution[37,39]; the remaining study reported no

Table 1 Summary of 45 case-control studies that evaluated the association between salt consumption and stomach cancer risk

Author and year	No. of cases	No. of controls	Factors evaluated	Exposure levels and OR (95% CI)[1]
SALT AND SODIUM INTAKE				
Modan *et al*, 1974: Israel[23]	166	429	Salt	No association
Risch *et al*, 1985: Canada[24]	246	246	Salt	No association
You *et al*, 1988: China[25]	564	1131	Salt (per capita household)	≤ 13 kg/yr, ≤ 19 kg/yr, ≤ 20 kg/yr, > 20 kg/yr 1.0, 1.2, 1.1, 1.1 (0.8-1.4); NS
Negri *et al*, 1990: Italy[26]	526	1223	Salt	Low, intermediate, salty 1.0, 1.3, 1.2 (0.8-1.7); NS
Wu-Willians *et al*, 1990: USA[27]	137 male	137	Add salt	Rarely, often, always 1.0, 1.4, 1.2 (Na); NS
Nazario *et al*, 1993: Puerto Rico[28]	136	151	Salt	≤ 6.979 g/wk; 6.98-18.66 g/wk; 18.67-43.26 g/wk; ≥ 43.27 g/wk 1.0, 2.9, 4.5, 5.0 (2.1-12.0); $P < 0.05$
Ramón *et al*, 1993: Spain[29]	117	234	Salt	Quartiles 1 through 4 1.0, 1.2, 1.8, 2.1 (1.2-7.1); P for trend < 0.01
Lee *et al*, 1995: Korea[30]	213	213	Salt	Tertile 3 *vs* 1 3.7 (1.1-12.5); $P < 0.05$
La Vecchia c *et al*, 1997: Italy[31]	746	2053	Salt	Low, intermediate or high 1.0, 1.5 (1.0-2.2); $P < 0.05$
Ye *et al*, 1998: China[32]	272	544	Salt	0.25 kg/m; > 0.25 kg/m 1.0, 1.3 (1.0-1.6); NS
López-Carrillo *et al*, 1999: Mexico[33]	220	752	Salt	Never, sometimes (adding salt after tasting the food) Positive association; $P < 0.05$
Liu *et al*, 2001: China[34]	189	189	Heavy salt	Low to high half 1.0, 2.0 (1.3-3.2); $P < 0.05$
Machida-Montani *et al*, 2004: Japan[35]	122	235	Salt	Tertiles 1 through 3 1.0, 1.3, 1.5 (0.6-3.7); NS
Qiu *et al*, 2004: China[36]	103	133	Salt	Positive association; $P < 0.05$
La Vecchia *et al*, 1987: Italy[37]	206	474	Table salt	Tertile 3 *vs* 1 1.5 (Na); NS
Tuyns *et al*, 1988: Belgium[38]	293	2851	Table salt	Never, sometimes, always 1.0, 1.0, 1.8 (1.2-2.8); $P < 0.05$
Buiatti *et al*, 1989: Italy[39]	1016	1159	Table salt	Seldom, always 1.5 (1.3-1.9); NS
Coggon *et al*, 1989: England[40]	95	190	Table salt	Low to high half 1.0, 6.2 (2.0-18.9); $P < 0.05$
Boeing *et al*, 1991: Germany[41]	143	579	Table salt	No association
Boeing *et al*, 1991: Poland[42]	741	741	Table salt	Low to high half 1.0, 1.6 (1.2-2.3); $P < 0.05$
Graham *et al*, 1990: USA[43]			Sodium intake	≤ 73.2 g/mo; 73.2-98.8 g/mo; ≤ 98.9-127.3 g/mo; > 127.3 g/mo
	186 male	181		1.0, 1.8, 2.6, 3.1 (1.7-5.8); P for trend = 0.001 ≤ 66.9; 67.0-88.5; > 88.5
	107 female	104		1.0, 1.8, 4.7 (2.3-9.6); P for trend = 0.0001
Harrison *et al*, 1997: USA[44]		132	Sodium intake	Low to high half
	60 intestinal			1.0, 1.3 (0.8-1.9); NS
	31 diffuse			1.0, 1.4 (0.9-2.1); NS
SALTY FOODS				
Salted fish				
Haenszel *et al*, 1972: Japan[45]	220	440	Salted/dried fish	None, use both, 2 times/mo; 3-5 times/mo; 6 times/mo 1.0, 2.0, 1.5, 2.5, 2.6 (Na); $P < 0.05$
Haenszel *et al*, 1976: Japan[46]	783	1566	Salted/dried fish	None; < 4 times/mo; 4-9 times/mo; ≥ 10 times/mo 1.0, 1.1, 1.1, 1.2 (Na); NS
Tajima *et al*, 1985: Japan[47]	93	186	Salted/dried fish	Low to high half 1.0, 2.6 (Na); $P < 0.01$
You *et al*, 1988: China[25]	564	1131	Sated fish	≤ 0.5 kg/yr, ≤ 1 kg/yr, > 1 kg/yr 1.0, 1.0, 1.4 (0.8-1.5); NS
Buiatti *et al*, 1989: Italy[39]	1016	1159	Salted/dried fish	Tertile 3 *vs* 1 1.4 (Na); P for trend = 0.001
Kato *et al*, 1990: Japan[48]				< 2-3 times/wk; ≥ 2-3 times/wk
	289 male	1247	Salted/dried fish	1.0, 1.2 (0.9-1.7); NS
			Salted fish gut, cod roe	1.0, 1.5 (1.1-2.1); $P < 0.05$
	138 female	1767	Salted/dried fish	1.0, 0.7 (0.5-1.0); NS
			Salted fish gut, cod roe	1.0, 0.5 (0.3-1.0); NS
González *et al*, 1991: Spain[49]	354	354	Salted fish	Low to high half 1.0, 1.5 (0.9-2.6); NS
Palli *et al*, 1992: Italy[50]		1159	Salted/dried fish	Tertile 3 *vs* 1

	68 cardia 855 others			1.7 (0.9-3.1); NS 1.5 (1.2-1.8); NS
Hansson *et al*, 1993: Sweden[51]	338	669	Salted fish	None, ⩽ 0.9 times/mo; ⩽ 3 times/mo; ⩽ 7 times/mo; ⩽ 11 times/mo 1.0, 1.0, 0.9, 0.9, 1.3 (0.8-2.1); NS (adolescence) None, ⩽ 0.9 times/mo; ⩽ 3 times/mo; ⩽ 7 times/mo 1.0, 1.0, 0.8, 0.8 (0.5-1.3); NS (20 yr prior to interview)
Kim *et al*, 2002: Korea[52]	136	136	Salted fish	Tertiles 1 through 3 1.0, 0.8, 0.8 (0.4-1.6); NS
Cai *et al*, 2003: China[53]	381	222	Salty fish	< times/mo, < 3 times/wk, ⩾ 3 times/wk 1.0, 1.0, 5.5 (1.4-19.5); NS
Strumylaite *et al*, 2006: Lithuania[54]	379	1137	Salted fish	Almost do not use, 1-3 times/mo 1.0, 0.7 (0.5-0.9); *P* for trend = 0.002
Salted vegetable				
You *et al*, 1988: China[25]	564	1131	Sated vegetables	< daily, daily 1.0, 1.1 (0.7-1.8); NS
Ye *et al*, 1998: China[32]	272	544	Salted vegetables	< 2 kg/yr; > 2 kg/yr 1.0, 1.4 (1.1-1.8); *P* < 0.05
Kim *et al*, 2002: Korea[52]	136	136	Salted vegetables	Tertiles 1 through 3 1.0, 0.9, 1.5 (0.8-2.9); NS
Xibin *et al*, 2002: China[55]	210	630		Low to high half
			Pickled or salted vegetables	1.0, 4.0 (1.6-9.8); *P* < 0.05
			Preference for a high salt vegetables	1.0, 2.6 (1.6-4.3); *P* < 0.05
Cai *et al*, 2003: China[53]	381	222	Pickled vegetables	< times/M, < 3 times/w, ⩾ 3 times/wk 1.0, 1.3, 1.8 (1.0-3.0); *P* for trend = 0.038
Strumylaite *et al*, 2006: Lithuania[54]	379	1137	Pickled vegetables with salt and oil	Almost do not use, 1-3 times/mo, ⩾ 1-2 times/wk
			Pickled vegetables with salt and vinegar	1.0, 0.6, 0.8 (0.6-2.1); NS
			Salted mushrooms	Almost do not use, 1-3 times/mo, ⩾ 1-2 times/wk 1.0,0.7, 0.8 (0.6-1.0); NS 1-3 times/mo, ⩾ 1-2 times/wk 1.0, 1.6 (1.1-2.4); NS
Salted snacks				
Boeing *et al*, 1991: Germany[41]	143	579	Pretzels, salty snacks	Tertiles 1 through 3 1.0, 0.7, 1.5 (1.0-2.2); NS
Ward *et al*, 1999: Mexico[56]	220	752	Salty snacks	Never, ⩽ 2, > 2 times/mo 1.0, 1.3, 1.8 (1.2-2.8); *P* for trend = 0.008
Chen *et al*, 2002: Nebraska[57]	124	449	Salty snacks	Quartiles 1 through 4 1.0, 1.4, 1.2, 0.7 (0.3-1.6);NS
Salted foods in general				
Hu *et al*, 1988: China[58]	241	241	Salted and fermented soya paste	< 2 kg/yr; > 2 kg/yr 1.0, 1.5 (1.0-2.2); NS
Kono *et al*, 1988: Japan[59]	139	2852	Salty foods	None or 1-3 times/mo; 1-3 times/mo; once/do more 1.0, 0.8, 1.4 (Na): NS
Demirer *et al*, 1990: Turkey[60]	100	100	Salted foods	Less than once or twice/wk; once or twice/wk *vs* 1.0, 3.8 (2.1-6.9); *P* < 0.001
Hoshiyama *et al*,1992: Japan[61]	294	294 (general population) 202 (hospital control)	Salty foods	No, moderate, yes 1.0, 1.7, 2.3 (1.5-3.4); *P* < 0.01 1.0, 1.3, 1.1 (0.7-1.9); NS
Ramón *et al*, 1993: Spain[29]	117	234	Pickled foods	Quartiles 1 through 4 1.0, 1.2, 2.1, 3.7 (Na); *P* for trend < 0.01
Ji *et al*, 1998: China[62]	1124	1451	Salted foods	Occasionally, sometimes, frequently 1.0, 1.4, 1.7 (1.3-2.4); *P* for trend = 0.001
Lee *et al*, 1995: Korea[30]	213	213	Salted side dishes	Tertile 3 *vs* 1 4.5 (2.5-8.0); *P* < 0.05
Ye *et al*, 1998: China[32]	272	544	Salted fermented sea foods	< 1.5 kg/yr; > 1.5 kg/yr 1.0, 1.6 (1.2-2.0); *P* < 0.01
Kim *et al*, 2002: Korea[52]	136	136	Salty foods	Tertiles 1 through 3 1.0, 1.1, 0.9 (0.4-1.8); NS
De Stefani *et al*, 2004: Uruguay[63]	240	960	Salted meat	Tertiles 1 through 3 1.0, 1.3, 2.0 (1.4-2.9); *P* for trend = 0.0003
Campos *et al*, 2006: Colombia[64]	368	431	Salting meals before tasting	No, yes 1.0, 3.5 (1.6-7.3); *P* for trend = 0.001
Strumylaite *et al*, 2006: Lithuania[54]	379	1137	Salted meat	Almost do not use, 1-3 times/mo, ⩾ 1-2 times/wk 1.0, 1.5, 3.0 (2.2-4.0); *P* for trend < 0.001

[1]OR, Odds ratio; CI: Confidence interval; Na: No association.

Table 2 Summary of 11 cohort studies that evaluated the association between salt consumption and stomach cancer risk

Author and yr	Size of cohort	No. of cases	Length of follow-up (yr)	Factors evaluated	Exposure levels and RR (95% CI)[1]
Salt					
Nomura *et al*, 1990: USA[65]	7990 male	150	4	Table salt/shoyu	Never-seldom, after tasting, always 1.0, 1.4, 1.0 (0.6-1.6); NS
van den Brandt *et al*, 2003: Netherlands[66]	120852	282	6.3	Dietary salt	Quintiles 1 through 5 1.0, 1.5, 1.0, 1.5, 1.2 (0.8-1.8); NS
				Table salt	Never, seldom, sometimes, often/very often 1.0, 1.1, 0.7, 0.9 (0.6-1.4); NS
Tsugane *et al*, 2004: Japan[67]			12	Salt	Quintiles 1 through 5
	18684	358			Male: 1.0, 1.7, 2.0, 2.3, 2.2 (1.5-3.4); *P* for trend < 0.001
	20381	128			Female:1.0, 0.9, 1.0, 0.6, 1.3 (0.8-2.3); NS
Shikata *et al*, 2006: Japan[68]	2476	93	14	Dietary salt	< 10.0, 10.0-12.9, 13.0-15.9, ≤ 16.0 1.0, 2.1, 1.9, 2.7 (1.4-5.2); *P* for trend = 0.01
Sjödahl *et al*, 2008: Sweden[69]	73133	313	18	Dietary salt	Low to high half 1.0, 1.0 (0.7-1.4); NS
Salty foods					
Kneller *et al*, 1991: USA[70]	17633 male	75	20	Salted fish	Never, < 1, ≥ 1 1.0, 1.0, 1.9 (1.0-3.6); NS
Galanis *et al*, 1998: USA[71]	11907	108	14.8 (average)	Dried or salted fish	None, 1 or more times/wk
				High-salt foods	1.0, 1.0 (0.6-1.7); NS None, 1-3 times/wk, 4 or more times/wk 1.0, 1.0, 1.1, (0.7-1.8); NS
Ngoan *et al*, 2002: Japan[72]	13000	116	10	Salted food	Low, median, high 1.0, 1.0, 1.4 (0.6-3.2); NS
Kim *et al*, 2004: Japan[73]		400	10	Salted food	Quartiles 1 through 4
	20300			(traditional type)	Male:1.0, 2.0, 2.5, 2.9 (1.8-4.7); *P* for trend < 0.0001
	21812				Female: 1.0, 1.7, 1.3, 2.4 (1.3-4.4); *P* for trend = 0.007
Tokui *et al*, 2005: Japan[74]	110792		12	Preference for salty food	No, a little, somewhat, much, very much
		574			Male: 1.0, 0.9, 1.1, 1.1, 1.4 (0.7-2.8); NS
		285			Female: 1.0, 1.6, 1.8, 1.5, 1.9 (0.6-5.8); NS
				Dried or salty fish	None, 1-2/mo, 1-2/wk, 3-4/wk, 1+/d
		574			Male: 1.0, 0.9, 0.9, 0.9, 1.1 (0.7-1.8); NS
		285			Female:1.0, 0.6, 0.7, 0.7, 0.9 (0.5-1.6); NS
Kurosawa *et al*, 2006: Japan[75]	8035	76	11	Salted food	Low, intermediate, high 1.0, 4.0, 5.4 (1.8-16.3); *P* for trend < 0.01

[1]RR: Relative risk.

association[41].

Of the twelve studies that estimated salted fish intake, four found strong statistically significant increases in risk (OR = 1.4-5.5 for the highest intake levels)[39,45,47,48]. One Japanese study reported a statistically significant increase in risk for high consumption of salted fish gut and cod roe in males, but not females, and no significant association for salted/dried fish for both genders[48]. Seven other studies reported statistically non-significant correlations[25,46,49-53]; the remaining study reported a statistically significant inverse association with odds ratios of 0.7 for consumption in the upper half of the salted fish intake distribution[54].

Six studies in Table 1 examined salted vegetables; of these, three reported statistically significant increases in risk with higher intakes of salted vegetables[32,53,55], the remaining three studies in China, Korea and Lithuania showed no relationship to stomach cancer risk[25,52,54]. Additionally, three studies reported on salted snacks. Of them, only one study in Mexico reported a statistically significant relationship to stomach cancer[56], with the other two reporting no substantial associations[41,57].

Twelve studies examined consumption of salted soya paste, salted side dishes and salty foods in general[29,30,32,52,54,58-64]. Nine of them observed a moderate increase in risk with higher consumption (OR = 1.6-4.5)[29,30,32,54,60-64], the remaining three reported no association[52,58,59].

In summary, many case-control studies found similar results, indicating a moderate to high increase in risk for the highest level of salt or salted food consumption. Given the large number of studies that reported data on salt, sodium and salty foods consumption, some inconsistent results were to be expected. The inconsistencies may be due, at least in part, to the retrospective assessment of salt exposure, which might have changed after the diagnosis of stomach cancer. Furthermore, the degree to which each of these measures reflects total salt intake varies, and it is therefore not surprising that results would vary.

Cohort studies

Eleven cohort studies, investigating salt or salted food consumption and stomach cancer risk in the US, Japan, Sweden, and the Netherlands have produced inconsistent results (Table 2)[65-75]. When viewed separately in the Table, the results were inconsistent for both salt intake and intake of salty foods. Four Japanese studies reported

statistically significant associations (range of RR = 2.2-5.4 for the highest intake level)[67,68,73,75], including one study that reported significantly elevated risks in both men and women after 10 years follow up of 20 300 men and 21 812 women[73]. Another study, conducted in rural Japan with 8035 subjects and 76 stomach cancer deaths, reported a significantly elevated relative risk for the most frequent intake of highly salted foods compared with the least frequent intake (RR = 5.4; 1.8-16.3; *P* for trend < 0.01)[75]. In a study that examined 18 684 men and 20 381 women and included 486 histologically confirmed stomach cancer cases (358 men and 128 women), there was a dose-dependent association between salt consumption and stomach cancer risk in men (*P* for trend < 0.001), but not in women (*P* for trend = 0.48)[67]. Shikata *et al*[68] categorized 2476 subjects into four groups according to daily salt intake. After 14 years of follow-up, the age- and sex-adjusted incidence was significantly higher in the second to fourth groups than in the first group (RR = 2.4, 95% CI: 1.2-4.7; RR = 2.1, 95% CI: 1.0-4.3; RR = 3.0, 95% CI: 1.5-5.8, respectively). With the exception of these positive findings, the remaining seven cohort studies showed no substantial associations[65,66,69-72,74]. It is perhaps noteworthy that four studies with statistically significant positive results were conducted in Japan, which may be related to a potentially higher range of salt intake in that country.

In summary, some cohort studies suggest that a higher intake of salt or of salted food, as estimated by validated food frequency questionnaires, may be directly associated (or at least indirectly linked) with subsequent development of stomach cancer. Although the overall results from cohort studies are not totally consistent, they are suggestive of a moderate direct association.

EVIDENCE ON INTERACTIONS BETWEEN SALT OR SALTED FOODS AND HELICOBACTER PYLORI INFECTION

Even though *H pylori* infection is the strongest risk factor for stomach cancer, it cannot completely explain the worldwide distribution of this disease. It is very important to evaluate the potential joint effects of *H pylori* infection and other factors, including salt intake, in stomach cancer carcinogenesis.

Few epidemiological studies have investigated *H pylori* infection in relation to salt consumption. In an international ecologic study[76], statistically significant correlations between national *H pylori* infection rates and national salt excretion levels were found in older (age 50-64) men and women (r = 0.73 and r = 0.83, respectively) and in younger (age 20-34) men (r = 0.73), but not in younger women (r = 0.52). A cross-sectional study of 634 Japanese men[77] reported that daily consumption of miso soup was associated with the prevalence of *H pylori* (OR = 1.60, *P*< 0.05). Similarly, increasing consumption of pickled vegetables was associated with increased *H pylori* infection risk (OR = 1.90 for the highest level, *P* for trend = 0.02). Despite limitations inherent in these types of studies, they can nevertheless provide information on potential associations between salted foods and *H pylori* infection in humans, which may be evaluated more fully in case-control and cohort studies.

Three previous epidemiological studies have examined the potential synergistic relationship between salt consumption and *H pylori* infection in the development of stomach cancer; however, the results are inconsistent[35,68,78]. A case-control study in Japan analyzing the independent and joint effects of diet and *H pylori* infection found that subjects with *H pylori* infection and with high salt intake (OR = 14.2) had a higher odds ratio compared with subjects with *H pylori* infection and low salt intake (OR = 9.7) (reference group was no *H pylori* infection and low salt intake), but there was not a statistically significant interaction between the two risk factors[35]. A Korean case-control study investigating the role of salt and *H pylori* infection in stomach cancer found that subjects with *H pylori* infection and high salt consumption had a 10-fold risk of early stomach cancer compared with subjects without *H pylori* infection and with a low salt consumption (*P* = 0.047)[78]. These two case-control studies have some limitations, including issues of possible recall bias and misclassification. For example, both *H pylori* and salt intake were assessed after the development of stomach cancer. Advancing stomach cancer can combine with the loss of infection characterized by a fall in circulating anti-*H pylori* antibodies and changes in salt exposure (or in recollection of dietary exposures prior to the onset of disease). Only one cohort study, conducted in Japan, evaluated the potential interaction between diet and *H pylori,* and found that the positive association between increased salt intake and gastric cancer was statistically significant among subjects with *H pylori* infection only[68]. The relative risks were similar, however, and the authors note that findings for dietary salt were most pronounced in subjects who had both *H pylori* infection and atrophic gastritis. The three studies discussed above have relatively small sample sizes, ranging from 69 to 122 stomach cancer cases, and thus the estimation of relative risk is imprecise and results should be interpreted cautiously, especially in the analyses of effect modification (interaction). Finally, the tendency of case-control studies to show stronger associations than cohort studies suggests the possibility that some degree of recall bias and/or selection bias may have influenced the results of the former.

Critical issues in interpreting salt consumption with stomach cancer risk

Most epidemiological data suggest an association between salt intake and the development of stomach cancer. When interpreting these data, several issues must be considered.

Assessment of salt intake is difficult and prone to some potential biases. Many commonly used dietary assessment methods, such as food frequency questionnaires and diet records, have reported only

moderate reproducibility in epidemiological studies, and thus some misclassification of dietary intake is inevitable[79,80]. This may be particularly true of total salt intake, given its nearly ubiquitous addition to most processed foods. In addition, use of different salt assessment methods may lead to different conclusions. For example, both 24 h urinary excretion and 3 d dietary record methods for salt intake estimation were used in one Japanese ecologic study. Only urinary salt excretion level showed a strong correlation with stomach cancer mortality, while dietary salt was weakly and non-significantly correlated[21]. Although 24 h urine collection may be an optimal method in estimating routine salt intake, it is impracticable for a large-scale population study, especially a cohort study with long-term follow-up.

Another critical issue in interpreting salt consumption in relation to stomach cancer risk is the variation in the salt consumption levels across the population. To date, there is no standard method for salt intake categorization, and several studies have reasonably compared categories, such as quintiles for the application in the specific study population. Because average salt intake varies across different populations, salt consumption levels considered "high" in one study might be considered "low" in another study. For example, in a study conducted in Japan, subjects reporting once per week consumption of salted food were in the lowest exposure category[48]; in contrast, the subjects in a Turkish study reporting once per week were classified into the highest exposure level (defined as $\geqslant$1-2 times/week)[60]. Finally, in some studies[75], salted food levels were calculated from the sum of the scores of foods belonging to the food items (pickled vegetable + foods deep-boiled in soy sauce), making it impossible to compare effects at similar levels of consumption across studies.

Because of the complexity of diets, the traditional approach with a single nutrient may potentially be confounded by the interactions between food components that are likely to be interactive or synergistic[81]. It is possible that the increased risks in stomach cancer could be due to compounds other than salt in foods that were produced during the preservation process[56]. In East Asia, salted foods and sauces are also high in NO_3, a chemical carcinogen, which may either be added to the foods or synergize from amino acids during fermentation. Nitrite and salt may work at an early stage[82] in a synergistic fashion on stomach cancer carcinogenesis[17] that might cause the strong associations between highly salted foods and gastric cancer[67]. However, nitrite was not clearly related with stomach cancer risk[83] and its function may be influenced by other factors. For example, when lower salt intake was combined with higher NO_3 intake, stomach cancer mortality rates tended to be lower[17]. However, this might be explained by a higher intake of fresh fruits and vegetables, which are the major source of nitrate and also protect against cancer[84,85].

Moreover, it may be difficult to separate the effects of salt from other nutrients that may contribute to stomach cancer risk. The absence of adjustment for confounding factors (such as age, sex, smoking and dietary habit) can hamper the statistical estimation causing over- or underestimation of the real association between salt or salted food and stomach cancer. In Tables 1 and 2, few studies have controlled for dietary factors in their analyses of salt consumption, which makes it difficult to compare the different studies according to the dietary variables adjusted in the analysis. However, the study results that were adjusted by a wide range of potentially confounding variables, such as age, sex, *H pylori* infection, atrophic gastritis, medical history of peptic ulcer, family history of cancer, body mass index, diabetes, total cholesterol, physical activity, alcohol intake, smoking habit and other dietary factors[68], showed no difference from the crude results. Studies with adjustment for some or most of the above potential confounding factors[66,68,72,73] showed no systematically apparent differences from the studies with adjustment for a few or several confounders.

BIOLOGICAL MECHANISMS

Several mechanisms by which salt intake may increase stomach cancer risk have been postulated, although to date there has been no consistent conclusion. High dietary salt intake may potentiate the colonization of *H pylori*[86], a known risk factor for stomach cancer, through the increase of surface mucous cell mucin and decrease of gland mucous cell mucin[87]. At the molecular level, high dietary salt intake may potentiate *CagA* (*H pylori* gene) expression and enhance the ability of *CagA* to translocate into gastric epithelial cells and enhance the ability of *H pylori* to alter gastric epithelial cell function[15]. Another explanation for the potential effect of high salt intake in gastric carcinogenesis is that high dietary salt intake helps to change the mucous viscosity protecting the stomach, potentiate exposure to carcinogens such as N-nitroso compounds, and lead to cell death[88]. In addition, high salt intake can cause damage to, and inflammatory responses of, the gastric epithelium[14], which may increase epithelial cell proliferation as part of the repair process and increase the probability of endogenous mutations[89,90]. One mechanism of high salt action in gastric carcinogenesis has been considered to induce hypergastrinemia in *H pylori*-infected gerbils[87]. Gastrin itself may mediate epithelial cell growth in *H pylori*-colonized mucosa[91] and chronic hypergastrinemia can synergize with *Helicobacter* infection and lead to eventual parietal cell loss and progression to gastric cancer[92].

ANIMAL STUDIES OF SALT AND STOMACH CANCER

Most published animal studies focus on the relationship between gastric cancer and several important suspected carcinogens, salt, *H pylori*, N-methyl-N-nitro-N-nitrosoguanidine (MNNG), and 4-nitroquinoline-1-oxide (NQO). In general, salt alone has no apparent

effect on the development of gastric carcinogenesis, but administration of salt in rats induced a concentration-dependent damage of surface mucous cell layer, and also increased replicative DNA synthesis[89]. Interestingly, a synergistic effect was observed when salt and other risk factors (*H pylori*, MNNG, NQO) were analyzed simultaneously. This conclusion was derived in animal experiments addressing gastric carcinogenesis from both the molecular level and tumor progression. At the molecular level, a high salt diet was associated with an elevation of anti-*H pylori* antibody titers, serum gastrin levels, and inflammatory cell infiltration in a dose dependent model in Mongolian gerbils infected with *H pylori*[87]. Similarly, in *H pylori* infected gerbils, a high-salt diet significantly up-regulated the expression of cyclooxygenase-2 (COX-2), and nitric oxide synthase (iNOS)[93]; the number of colony-forming units was also significantly higher. Dietary sodium chloride also produced a reduction in cell yield, and an increase in S-phase cell numbers that are the most susceptible to mutagenesis, which may possibly increase tumor incidence[90]. Several studies examined gastric tumor progression in mice infected with *H pylori* and administered with a high-salt diet, and all of these studies consistently demonstrate that a high-salt diet enhances the effects of *H pylori* infection, and consequently promotes the development of stomach cancers[88,94,95]. Additionally, a high-salt diet significantly increased gastric tumor incidence in those mice pre-treated with MNNG[14] or MNU[96], suggesting that salt and chemical carcinogens also exert a synergistic effect in the development of gastric carcinogenesis.

CONCLUSION

Most published epidemiological studies provide positive evidence for an association between salt or salted food consumption and stomach cancer risk, which was also supported by experimental studies[14,87,94,97]. The limitations of salt assessment in epidemiological studies may have attenuated the true effect of salt intake on stomach cancer risk, or even biased the results away from the null, in the reviewed ecological, case-control, and cohort studies. Ideally, dietary modification of salt intake, as well as eradication of *H pylori* infection, is a promising strategy for gastric cancer prevention throughout the world, especially in developing counties. However, the former strategy is more practical than the latter according to previous epidemiological studies. Future studies that address the association with salt and other dietary factors and the interactions between these factors in different aspects, e.g. molecular level, may help to shed light on the etiology of stomach cancer.

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ORIGINAL ARTICLES

Feasibility of confocal endomicroscopy in the diagnosis of pediatric gastrointestinal disorders

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Abstract

AIM: To evaluate the feasibility and utility of confocal laser endomicroscopy (CLE) in the description of normal gastrointestinal (GI) mucosa and in the diagnosis of GI disorders in children, in comparison to histology.

METHODS: Forty-four patients (19 female) median age 10.9 years (range 0.7-16.6 years) with suspected or known GI pathology underwent esophago-gastro-duodenoscopy (OGD) (n = 36) and/or ileocolonoscopy (IC) (n = 31) with CLE using sodium fluorescein and acriflavine as contrast agents. Histological sections were compared with same site confocal images by two experienced pediatric and GI histopathologists and endoscopists, respectively.

RESULTS: Duodenum and ileum were intubated in all but one patient undergoing OGD and IC. The median procedure time was 16.4 min (range 7-25 min) for OGD and 27.9 min (range 15-45 min) for IC. A total of 4798 confocal images were compared with 153 biopsies from the upper GI tract from 36 procedures, and 4661 confocal images were compared with 188 biopsies from the ileocolon from 31 procedures. Confocal images were comparable to conventional histology both in normal and in pathological conditions such as esophagitis, *Helicobacter pylori* gastritis, celiac disease, inflammatory bowel disease, colonic heterotopia, and graft versus host disease.

CONCLUSION: CLE offers the prospect of targeting biopsies to abnormal mucosa, thereby increasing diagnostic yield, reducing the number of biopsies, decreasing the burden on the histopathological services, and reducing costs.

Key words: Confocal laser endomicroscopy; Histology; Pediatric; Gastrointestinal mucosa; Gastrointestinal disorders

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INTRODUCTION

Modern endoscopy has recently seen the development of technological advances with the aim of increasing and optimizing diagnostic yield from the procedure. These have included video and magnification endoscopes[1]. Greater surface definition has been achieved with chromo-endoscopy, and recently, narrow-band imaging has allowed greater definition of vascular architecture[2-4]. However *in vivo* sub-surface pathology remained obscure to the endoscopist until the advent of confocal endomicroscopy, which affords magnification up to

1000 ×, and with sequentially deeper images from the epithelial surface to approximately 250 μm below the surface. This allows histological assessment of the *in vivo* gastrointestinal (GI) mucosal structure at the cellular and subcellular level[5,6]. In addition, this technique avoids crush artefacts from the grasp biopsy forceps and changes from histopathological processing.

The diagnosis of upper GI disorders in children depends to a great extent on endoscopy and subsequent histology of biopsy specimens[7]. Pathology such as gastroesophageal reflux disease (GERD)[8-10], eosinophilic esophagitis (EE)[11,12]; *Helicobacter pylori (H pylori)* gastritis[13,14], and celiac disease (CD)[15,16], in conjunction with various other investigative modalities have, as pivotal to their diagnosis, histological confirmation. Similarly, pediatric ileocolonic conditions such as inflammatory bowel disease (IBD)[17,18], familial adenomatous polyposis (FAP), graft versus host disease (GVHD)[19,20], and allergic colitis[21,22] necessitate a tissue diagnosis.

The aims of this study were to evaluate the feasibility and utility of confocal laser endomicroscopy (CLE) in the description of confocal features of normal GI mucosa and in the diagnosis of GI disorders in children.

MATERIALS AND METHODS

Patients

Forty-four patients with a potential diagnosis of GI pathology that required upper GI endoscopy and/or ileocolonoscopy as part of the clinical management were enrolled in the study. Written informed consent was obtained from parents and, where age and competency were appropriate, from each patient, before the examination. The study protocols were reviewed and approved by South Sheffield Regional Ethics Committee. Patient exclusion criteria were as follows: inability to give signed informed consent; age > 18 years; previous documented adverse reaction/allergy to sodium fluorescein or acriflavine hydrochloride; and non-correctable coagulopathy (PT > 14 s/platelet count < 90 000). The study was conducted between December 2005 and July 2007 at Sheffield Children's Hospital NHS Foundation Trust.

Indications for upper GI endoscopy alone included: children with suspected GERD; Barrett's esophagus; suspected peptic ulcer disease; suspected celiac disease based on raised anti-endomysial and tissue transglutaminase antibodies; and non-specific recurrent upper abdominal pain. Indications for ileocolonoscopy included: chronic diarrhea; presence of fecal blood; recurrent abdominal pain; weight loss; mutation of the APC gene; colonic heterotopia; and suspected GVHD.

Forty-four patients (19 female) with a median age of 10.9 years (range 0.7-16.6 years), and a median weight of 41.5 kg (range 8-97 kg) with suspected or known GI pathology were enrolled.

Patients undergoing ileocolonoscopy were admitted the previous day and had bowel preparation as for standard ileocolonoscopy. Patients undergoing upper GI endoscopy were admitted on the day of the procedure. All procedures occurred under general anesthesia, as is normal practice in our institution for pediatric GI endoscopy.

CLE

CLE involves the use of a highly miniaturized confocal microscope that has been incorporated into the distal tip of a flexible endoscope to allow *in vivo* microscopic examination of the gut mucosa. The confocal microscope uses a single optical fiber to deliver 488 nm laser light to the distal tip of the endoscope, where it is focused to a single diffraction-limited point within the tissue. The laser light excites fluorescent molecules within the tissue. Fluorescent light emanating from the specific point of focus is collected into the same optical fiber of the confocal microscope and delivered to the photodetector. Light emanating from outside the focally illuminated spot is not focused into the optical fiber and therefore, is geometrically rejected from detection. The focused point of laser light is scanned in a raster pattern across the field of view, and the intensity of the fluorescent signal returning to the detector from successive points is measured (12-bit digitization) to produce two-dimensional images that are *en face* to the tissue surface. By moving the microscope optics within the confocal microscope, the operator can dynamically adjust the imaging depth to allow microscopic imaging at and below the surface of the mucosa; hence each image is an optical section representing one focal plane within the specimen[5,23], and collection of multiple optical sections at successive depths results in true volumetric sampling of the tissue. As a three-dimensional volume is thus sampled, this can be thought of as a virtual biopsy.

The Pentax EC3870CILK endoscope has a 5-mm diameter miniaturized confocal microscope integrated into the distal tip of the endoscope. The diameter of the distal tip and insertion tube of the endoscope is 12.8 mm. In addition to the integrated confocal microscope, the distal tip also contains a color CCD camera which enables simultaneous confocal microscopy with standard video-endoscopy, air and water jet nozzles, two light guides, a 2.8-mm working channel, and an auxiliary water jet channel. During CLE, the laser delivers an excitation wavelength of 488 nm at a maximum laser output of 1 mW to the tissue (typically 300-700 μW). Confocal images can then be collected at either 1024 × 1024 pixels (0.8 frames/s) or 1024 × 512 pixels (1.6 frames/s). The optical sections have a 475 μm × 475 μm field of view, with a lateral resolution of 0.7 μm, axial resolution of 7.0 μm, and an imaging depth (z axis) range of 0-250 μm below the tissue surface, in 4-μm steps. The imaging depth below the tissue surface can be dynamically controlled by the operator. CLE magnifies images 1000-fold.

Contrast agents

Fluorescein sodium (FS) 10% and acriflavine hydrochloride (AH) 0.05% were used as contrast agents. FS is highly water-soluble and, on intravenous administration, rapidly diffuses in seconds from the

capillaries into the extra-vascular tissue. FS, when exposed to light of wavelength 465-490 nm (blue), emits light at longer wavelengths (520-650 nm, with the peak emission in the 520-530 nm green-yellow region)[24]. This enables visualization of microvessels, cells and connective tissue. However FS is not enriched in the nuclei of intestinal epithelial cells, and hence, the nuclei are not readily visible in the confocal images. To circumvent this limitation, AH (0.05%) is used topically to enrich the superficial nuclei and to a lesser extent the cytoplasm.

CLE was performed by a single experienced endoscopist (MT), who had completed the Mainz CLE training program prior to patient recruitment, using the confocal laser endomicroscope (EC3870CILK; Pentax, Tokyo, Japan). Ten to twenty milligrams Buscopan (hyoscine-N-butyl-bromide; Boehringer, Ingelheim, Germany) was given intravenously to limit peristaltic artefacts. Following duodenal or ileal intubation, 0.05-0.1 mL/kg of 10% FS was administered intravenously and flushed adequately with normal saline. AH (0.05%) was applied to the mucosa using a spray catheter at all sites undergoing confocal imaging.

CLE image acquisition was performed by placing the tip of the colonoscope in direct contact with the target tissue site. Using gentle suction to stabilize the mucosa, image acquisition and focal plane z-axis scanning depth was then actuated using two discrete hand-piece control buttons. Confocal images were sequentially obtained from a third part of the duodenum, gastric antrum and body, and distal and proximal esophagus in the upper GI tract, and ileum, cecum, ascending, transverse, descending and sigmoid colon, and rectum in the ileocolon region. Confocal images were acquired simultaneously with ongoing video endoscopic imaging. Same site mucosal specimens were obtained using standard biopsy forceps. The biopsy specimens were fixed in buffered formalin solution, embedded in paraffin wax, and serial sections were obtained and stained with hematoxylin and eosin (HE). The histological specimens from each site were compared with same-site confocal images jointly by the endoscopists and two experienced pediatric and GI histopathologists (MC, CE).

RESULTS

Twenty-three patients underwent both upper GI and ileocolonoscopy, while 13 had upper GI endoscopy and eight had ileocolonoscopy alone. The youngest patient 8 mo of age, with suspected GVHD, had proctoscopy alone. The duodenum at upper GI endoscopy and the terminal ileum at ileocolonoscopy were intubated in all patients, except for one who weighed 10 kg, in whom the pylorus and ileocecal valve were both too narrow to accept the confocal endomicroscope. The youngest and smallest patient to have full successful examinations up to a third part of the duodenum and terminal ileum was 18 mo old and weighed 11 kg. The procedure time was 7-25 min (median 16.4 min) for upper GI endoscopy, and 15-45 min (median 27.9 min) for ileocolonoscopy. A total of 153 pinch biopsies were taken from the upper GI tract from 36 procedures and 188 from the ileocolon from 31 procedures.

No complications or adverse effects occurred, except on one occasion when precipitation was observed in the peripheral venous line when fluorescein was injected immediately after neostigmine.

Upper GI tract

Thirty-six patients underwent upper GI endoscopy. The duodenum was intubated in all patients except one. A total of 4798 confocal images were obtained, which included 2010 from the duodenum, 1616 from the stomach and 1172 from the esophagus, and were compared with 153 biopsies.

On confocal imaging, the duodenal villi had a long, slender and finger-like appearance (Figure 1A) similar to that in histological specimens (Figure 1C). The single layer of brush border columnar epithelial cells interspersed with intraepithelial lymphocytes and goblet cells was clearly visible. Crypts were not normally visible except in the presence of villous atrophy.

The gastric pits or foveolae appeared as invaginations on the surface epithelium (Figure 1B and D). Each confocal image showed several evenly spaced such pits lined by columnar epithelium. The center of the pits appeared dark.

The esophagus was lined by non-keratinized squamous epithelium with polygonal epithelial cells. The nuclei of the epithelial cells were highlighted clearly following topical administration of acriflavine (Figure 1C and F). Furthermore, the capillary loops in the papillae were visible in deeper planes following subsurface optical sectioning, and surface to capillary distance could be measured as each level was deeper by 4 μm. This allowed assessment of GERD-like histopathology, given that papillary height was increased and epithelial surface to papillary tip (i.e. where capillary loops appeared on confocal endomicroscopy) distance was thereby shorter.

Lower GI tract

A total of 31 patients underwent ileocolonoscopy. Two patients had only proctoscopy, while total ileocolonoscopy was performed in the rest, with the terminal ileum intubated in all but one patient, who weighed 10 kg. A total of 4661 confocal images, which included 945 from the terminal ileum, 2919 from the colon and 797 from the rectum, were compared with 184 biopsies.

The confocal appearance of the normal ileum and colon in adults has been described previously[25]. The villi in the terminal ileum appeared similar to those in the duodenum. Colonic architecture on confocal imaging showed numerous evenly distributed crypts lined by columnar-shaped enterocytes (Figure 2A and B). The luminal openings of the crypts appeared as black holes in the horizontal axis. The mucin-containing goblet cells were readily identified and appeared dark. At deeper planes, the vessel architecture had a hexagonal, honeycomb pattern, which represented a network of capillaries that outlined the stroma surrounding the

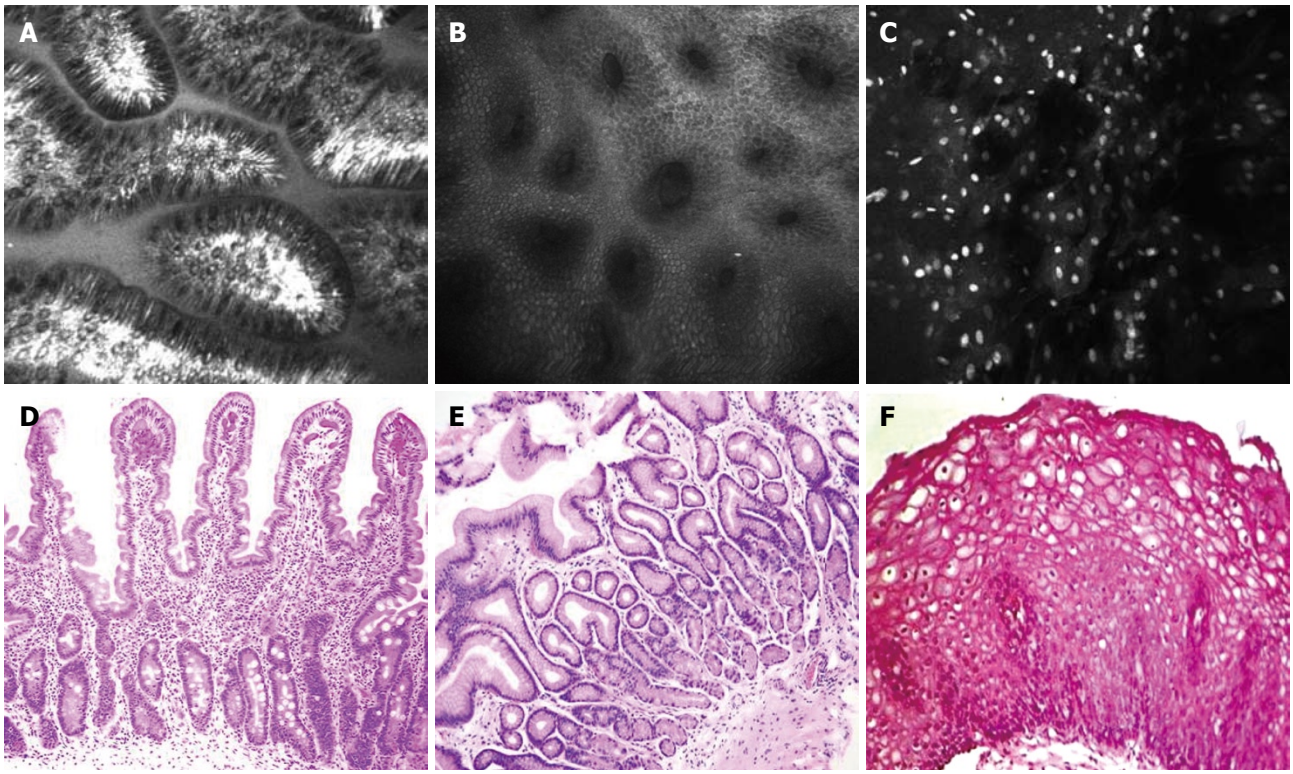

Figure 1 Comparison of confocal images with conventional histological images of the upper GI tract. A: Confocal image delineating the fine slender fingerlike projections of the duodenal villi; B: Confocal image showing gastric pits; C: Confocal image of non-keratinized squamous epithelium of the esophagus; D: Histological image of duodenum; E: Histological image of gastric antrum; F: Histological image of esophagus.

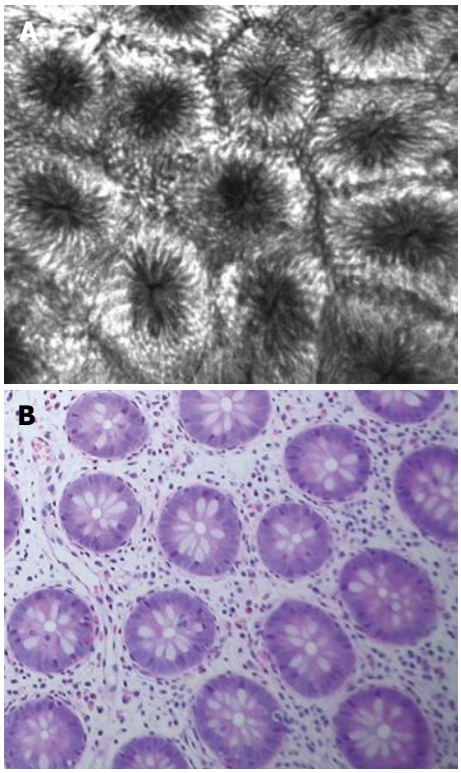

Figure 2 Comparison of confocal images with conventional histological images of the lower GI tract. A: Confocal image of normal colonic mucosa showing regularly spaced crypts; B: Comparative histological image.

luminal openings of the crypts. Individual red cells were also visible as black dots in the lumen of the capillaries.

Confocal imaging in GI pathology

Upper GI pathology: Two patients had histologically proven esophagitis. At CLE, capillary loops were visible at about 24 and 44 μm below the surface epithelial layer, which indicated the presence of papillae. In comparison, capillary loops were seen at a median of 72 μm (range 48-100 μm) from the surface of the esophageal mucosa in those without histologically proven esophagitis.

One patient with suspected *H pylori* upon endomicroscopy of the gastric antrum showed multiple focal lesions that resembled focal accumulation of *H pylori*, which was subsequently confirmed by Campylobacter-like organism test and histology. These lesions were demonstrated both on the surface of the epithelium and deeper in the crypt lumen.

Four patients had a histological diagnosis of celiac disease (Figure 3A and C). Three patients had Marsh type 3b with marked villous atrophy, increased intra-epithelial lymphocytes and crypt hyperplasia. CLE features (Figure 3B) in these patients were as follows: (1) increased basal width of villi; (2) gross distortion of the cellular architecture of the villous epithelium with loss of the honeycomb pattern; (3) damaged villous border; (4) "sticky" villi with inter-villous bridging; and (5) in-folding of villi. One patient had total villous atrophy. Confocal imaging revealed absence of villi with crypt hyperplasia (Figure 3D).

Lower GI pathology: Seven patients had a histological diagnosis of IBD, including three patients each with ulcerative colitis and Crohn's disease, and one with

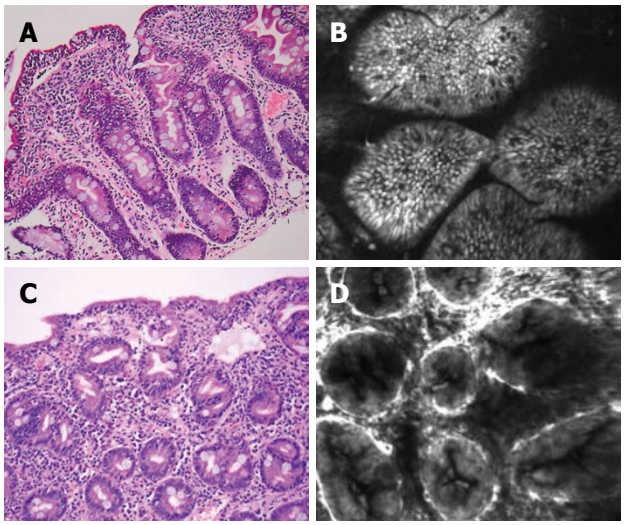

Figure 3 Comparison of confocal with conventional histology in celiac disease. A: Histological image of celiac disease, Marsh type 3b; B: Comparative confocal image; C: Histological image of celiac disease, Marsh type 3c; D: Comparative confocal image.

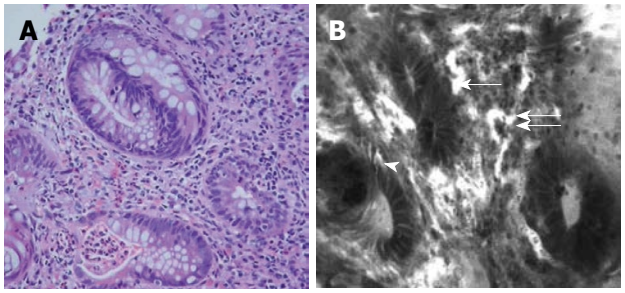

Figure 4 Comparison of confocal with conventional histology in ulcerative colitis. A: Histologic image in Ulcerative colitis; B: Comparative confocal image showing bifid crypt (arrow), crypt destruction (arrow head), tortous vessels (double arrows).

indeterminate colitis. Features of IBD seen on confocal imaging included bifid crypts, crypt distortion and destruction, crypt abscess/cryptitis goblet cell depletion and inflammatory cell infiltration, enlarged tortuous vessel architecture (Figure 4B), and comparable to histology (Figure 4A).

Other lower GI pathologies: Two patients with suspected GVHD following bone marrow transplantation underwent proctoscopy. Apoptotic nuclei were visualized during confocal imaging. This was confirmed on histology of biopsy specimens. A rare case of colonic heterotopia that presented with persistent diarrhea and had large tracts of abnormal looking mucosa on endoscopy, showed squamous, gastric and small-intestinal mucosal features on confocal imaging. Histology confirmed the presence of aberrant mucosa.

DISCUSSION

A definitive diagnosis of GI disorders in children usually requires GI endoscopy and histology of biopsy tissue. Technological innovations have led to the development of chromo-endoscopy, for which dyes such as methylene blue and indigo carmine are used to aid localization of lesions, and magnifying endoscopy has enabled visualization of surface structures at approximately × 100 magnification. In adults, several studies have validated these techniques in differentiating neoplastic from non-neoplastic lesions[26-29], diagnosis of neoplastic lesions in flat and depressed lesions in the colorectum, and in cancer surveillance in patients with long-standing ulcerative colitis[30,31]. Confocal endomicroscopy is a newly developed tool that enables surface and subsurface imaging of living cells in the mucosa during ongoing endoscopy. It offers the combination of video endoscopy and confocal endomicroscopy, which uniquely provides *in vivo* histology and what might be termed a virtual biopsy[6]. The confocal endomicroscopy images obtained are in a single optical plane parallel to the surface of the tissue. Collection of multiple optical sections at successive depths allows detailed visualization of successive tissue layers, and allows sampling of a three-dimensional volume of tissue. This is in contrast to conventional histology in which the tissue is sectioned vertically, making it possible to see all the tissue layers in one view using a bench top light microscope. Hence, it is pertinent that confocal images require comparison with similarly sectioned histological images. The endoscopist also requires training in normal and abnormal microscopic anatomy, which takes time. Also a certain amount of training in using the endomicroscope and interpreting the image data is necessary.

In this study, the feasibility of CLE in the diagnosis of GI disorders was determined in children as young as 8 mo of age and as light as 10 kg, for the first time. Confocal findings of normal GI mucosa are described. In addition, confocal features in conditions such as pediatric manifestations of GERD, *H pylori* gastropathy, celiac disease, IBD, GVHD, and colonic heterotopia were illustrated.

The tantalizing prospect of targeted biopsies or even a biopsy-free endoscopic procedure in the diagnosis of childhood GI disorders arises, with obvious potential benefits in terms of avoidance of biopsy-associated complications, and diminution of the considerable histological burden that this patient cohort places on already over-stretched histopathological services, along with the prospect of considerable associated cost savings.

COMMENTS

Background

Histology of biopsy specimens has a major role in the definitive diagnosis of pediatric gastrointestinal (GI) disorders, but is subject to changes from crush artefacts and processing, in addition to an inherent delay in diagnosis. Confocal laser endomicroscopy (CLE) is a recent development that enables surface and subsurface imaging of living cells *in vivo* at × 1000 magnification.

Research frontiers

The relatively new tool of CLE has been used in the assessment and *in vivo* diagnosis of various GI disorders in adults, such as Barrett's esophagus, esophageal, gastric and colorectal cancers, and inflammatory bowel disease and other forms of colitis.

Innovations and breakthroughs

This is believed to be the first study to assess this innovation in the diagnosis of pediatric GI disorders. This study confirmed the feasibility and diagnostic

reliability of CLE for the *in vivo* diagnosis of a wide range of GI disorders in childhood.

Applications

This study provides a basis for future studies on the use of this advanced diagnostic technique in providing a real-time diagnosis during endoscopy in children.

Peer review

This was a well-conducted study, which shows that it is feasible to use confocal endomicroscopy in the diagnosis of childhood GI disorders.

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ORIGINAL ARTICLES

Intestinal microflora molecular markers of spleen-deficient rats and evaluation of traditional Chinese drugs

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Abstract

AIM: To find a rapid and efficient analysis method of gastrointestinal microflora in Pi-deficient (spleen-deficient) rats and to evaluate traditional Chinese drugs.

METHODS: Enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR) based assay was performed to examine changes of intestinal microflora in two Pi-deficienct animal models and to evaluate the efficacy of four traditional Chinese drugs as well as a probiotic recipe and another therapy in Pi-deficient rats.

RESULTS: A molecular marker was identified for Pi-deficiency in rats. The pharmacodynamic evaluation system, including identified molecular markers (net integral area and abundance of DNA bands), Shannon's index for diversity of intestinal microflora, and Sorenson's pairwise similarity coefficient, was established. The four major clinical recipes of traditional Chinese drugs for Pi-deficiency in rats, especially at their medium dose (equivalence to the clinical dose), produced more pronounced recovery activities in Pi-deficient rats, while higher doses of these recipes did not show a better therapeutic effect but some toxic effects such as perturbation deterioration of intestinal microflora.

CONCLUSION: Both fingerprint analysis and identified marker can show Pi-deficiency in rats and its difference after treatment. The identified molecular marker may be applied in screening for the active compounds both in relative traditional Chinese drugs and in pharmacodynamic study of Pi-deficiency in rats.

Key words: Pi-deficiency; Enterobacterial repetitive intergenic consensus-PCR; Traditional Chinese medicine

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INTRODUCTION

Pi-deficiency (spleen deficiency), a common clinical syndrome in traditional Chinese medicine (TCM), is described as symptoms such as epigastralgia, flatulence after meal, lack of appetite, wilted complexion, loose stool, lassitude, fatigue, *etc.* The "Pi" here is the Chinese spelling of "spleen" in TCM, which relates to the functions of digestion, absorption and nutrition, differs from the "spleen" in Western medicine that belongs to the blood and immune systems. Pi-deficiency in TCM is one of the most common digestive diseases and usually the patients' equilibrium of gastrointestinal microflora is broken, which plays an important role in the growth, development and performance of the host[1]. Therefore, more clinical interests are arising in monitoring changes of intestinal microflora in intestinal disease and its consequent treatment with TCM therapies. It has been found that some traditional Chinese drugs have curative effects on Pi-deficiency by regulating the equilibrium of intestinal microflora and therefore promote the recovery of Pi-deficiency[2-4].

However, methods of monitoring the intestinal flora are quite limited, not only because of the complexity of

its constitution, but also the difficulty in culturing most gastrointestinal bacteria *in vitro*. Recent development in molecular biology techniques provides various possibilities of illustrating microbial biodiversity without *in vitro* culture of bacteria[5-6]. Enterobacterial repetitive intergenic consensus (ERIC) sequences are non-coding sequences of highly conserved 127 bp that are repeated multiple times through the genome of most bacterial species[7]. Variation in the number and location of ERIC sequences between different populations of microbes will result in differences between strains in the number and size of PCR products by ERIC primers. Based on this, ERIC-PCR has been used to investigate the diversity of bacteria[8-11].

In this study, we introduced ERIC-PCR fingerprinting in study of Pi-deficiency syndrome, used molecular markers to detect changes of intestinal microflora in Pi-deficient rats, and evaluated the therapeutic effects of traditional Chinese drugs.

MATERIALS AND METHODS

Animals

Wistar rats (200 ± 20 g) of either sex were obtained from the Experimental Animal Centre of Shenyang Pharmaceutical University (Shenyang, China). The rats were kept under standard environmental conditions with free access to rodent diet and water. All animal experiments were performed in accordance with the Guidelines for Use of Experimental Animals established by Shenyang Pharmaceutical University.

Plant materials

Plant materials including *Radix* and *Rhizoma Rhei*, *Folium Sennae, etc,* used in the study, were purchased from a local TCM apothecary in Shanghai, China (Table 1), and identified by Dr. Meng-Yue Wang, Department of Pharmacognosy, School of Pharmacy, Shanghai Jiao Tong University.

Preparation of traditional Chinese drug decoctions

One hundred milliliters aqueous decoction was prepared with 100 g of each crude *Radix, Rhizoma Rhei* and *Folium Sennae*. For the preparation of decoction of traditional Chinese drug recipes, the crude drugs were mixed first according to the ratio as prescribed, and then decocted 3 times in 10 volumes of distilled water for 30 min, finally the solution was filtered and concentrated. The ratios and concentrations are shown in Table 1.

Induction of Pi-deficiency in rats and treatment with traditional Chinese drugs

Rats were randomly divided into 16 groups (n = 8). Rats in group 1 received distilled water only (10 mL/kg, *po*) during the whole experiment. Rats in groups 2-16 were intragastrically given *Radix* and *Rhizoma Rhei* extract, 10 mL/kg, twice a day for the first 10 d to induce Pi-deficiency[12]. Rats in group 2 (model group) received distilled water only, once a day for 10 d. Rats in group 3 received Entrocoordinatibiogen (16.2 mg/kg, *po*), once a day for 10 d. Rats in group 4 received Banxia Houpu Tang (Decoction of Pinellia and Magnolia Bark, 4.3 g crude drug/kg, *po*), once a day for 10 d. Rats in groups 5-7 were treated with Si Junzi Tang (Decoction of Four Noble Drugs, 1.2, 3.5 and 10.5 g of each crude drug/kg, *po*), once a day for 10 d. Rats in groups 8-10 received Lizhong Tang (Decoction for Regulating the Function Of Middle Jiao, 1.8, 5.4 and 16.2 g of each crude drug/kg, *po*), once a day for 10 d. Rats in groups 11-13 received Buzhong Yiqi Tang (Decoction for Regulating the Function Of Middle Jiao and Supplementing Qi, 1.6, 4.8 and 14.4 g of each crude drug/kg, *po*), once a day for 10 d. Rats in groups 14-16 received Yiwei Tang (Decoction for Nourishing the Stomach, 1.9, 5.8 and 17.4 g of each crude drug/kg, *po*), once a day for 10 d. Banxia Houpu Tang is a recipe for tussis but not for Pi-deficiency. Si Junzi Tang, Lizhong Tang, Buzhong Yiqi Tang and Yiwei Tang are commonly used clinical recipes for Pi-deficiency. Another experiment was performed as described above, except that 10 mL/kg *Folium Sennae* was given instead of *Radix and Rhizoma Rhei* to induce Pi-deficiency[13,14].

Sample collection and total DNA extraction

Three or four pieces of fecal pellets (about 1 g) per rat were directly collected from the anus into sterile plastic tubes and stored at -20℃ immediately. Fecal pellets were collected 5 d before induction of Pi-deficiency and then every two days.

Total DNA was isolated from the fecal samples as previously described[10] with some modifications. Each sample (0.2 g) was suspended in 1 mL sterile 0.05 mol/L PBS (pH 7.4) followed by vortexing for 5 min in a 2 mL tube. The suspension was centrifuged at 200 × g for 6 min and the supernatant was transferred to a new tube. Then 1 mL sterile PBS was added to the pellets and vortexed for 5 min, the suspension was centrifuged and the supernatant was transferred to the new tube as well. Combination of the two sets of supernatant was then centrifuged at 300 × g for 6 min to remove coarse particles. The cells in the supernatant were collected and washed twice with PBS by centrifuging at 10000 r/min for 6 min. The washed cell pellets were resuspended in 300 μL of solution Ⅰ containing 150 mmol/L NaCl, 50 mmol/L Na_2EDTA (pH 8.0). The suspension was gently mixed with 100 μL lysozyme solution (100 mg/mL) and 20 μL RNase (10 mg/mL), pre-warmed in 37℃ water bath for 30 min and then combined with 300 μL of solution Ⅱ containing 100 mmol/L NaCl, 50 mmol/L Tris base (pH 8.0). The cell suspension was gently mixed with 100 μL of 10% SDS and 50 μL of 20% PVP, and incubated on ice for 5 min. DNA was then purified by sequential extraction with Tris-equilibrated phenol and chloroform-isoamyl alcohol (v/v/v, 25:24:1), and chloroform isoamyl alcohol (v/v, 24:1) followed by precipitation with 2 volumes of ethanol and 50 μL of 3 mol/L sodium acetate. DNA was collected by centrifugation and washed once with 70% ethanol,

Table 1 Clinical recipes of traditional Chinese drugs used in this study

Prescription	Composition	Dose (g crude plants/kg)	Effect[a]
Banxia Houpu Tang	*Rhizoma Pinelliae*, *Poria*, *Cortex Magnoliae Officinalis*, *Folium Perillae*, *Rhizoma Zingiberis Recens* (4:4:3:3:2)	4.3 (clinical dose)	N↓, A↓, C↓, H↓
Si Junzi Tang	*Radix Ginseng*, *Rhizoma Atractylodis Macrocephalae*, *Poria*, *Radix Glycyrrhizae* (10:9:9:6)	1.2 (triplicate of clinical dose)	N↓[1], A↓[1], C↑, H↑
		3.5 (clinical dose)	N↓[1], A↓[1], C↑[1], H↑
		10.5 (triplication of clinical dose)	N↓[1], A↓, C↑, H↓
Lizhong Tang	*Radix Codonopsis*, *Rhizoma Atractylodis Macrocephalae*, *Rhizoma* Zingiberis, *Radix Glycyrrhiza preparata* (1:1:1:1)	1.8 (triplicate of clinical dose)	N↓, A↓, C↑, H↑
		5.4 (clinical dose)	N↓[1], A↓[1], C↑, H↑
		16.2 (triplication of clinical dose)	N↓[1], A↓, C↑, H↑
Buzhong Yiqi Tang	*Radix Astragali*, *Radix Ginseng*, *Radix Angelicae Sinensis*, *Rhizoma Atractylodis Macrocephalae*, *Radix Glycyrrhiza preparata*, *Radix Bupleuri*, *Rhizoma Cimicifugae*, *Pericarpium Citri Reticulatae* (6:1:1:1:3:2:2:2)	1.6 (triplicate of clinical dose)	N↓[1], A↓[1], C↑, H↑
		4.8 (clinical dose)	N↓[1], A↓[1], C↑[1], H↑
		14.4 (triplication of clinical dose)	N↓[1], A↓, C↑, H↓
Yiwei Tang	*Radix Glehniae*, *Raidix Ophiopogonis*, *Rehmannia Dride Rhizome*, *Rhizoma Polygonati Odorati*, rock candy (3:3:3:1:3)	1.9 (triplicate of clinical dose)	N↓[1], A↓[1], C↑, H↓
		5.8 (clinical dose)	N↓[1], A↓[1], C↑, H↓
		17.4 (triplication of clinical dose)	N↓, A↓, C↑, H↓

[a]N: Net area of 380 bp; A: Abundance of 380 bp; C: Sorenson's pairwise similarity coefficient (Cs); H: Shannon's index (H'); ↑: Increase; ↓: Decrease; [1]: Significant ($P < 0.05$).

air dried and dissolved in 50 μL of sterile distilled water. The DNA was checked for integrity first by electrophoresis analysis on 1% agarose gel (compared with size-known Hind Ⅲ digested bacteriophage λ DNA), and then quantified.

ERIC-PCR

ERIC-PCR was performed on a MJ Research PTC-100 thermal cycler (MJ Research, Inc., Waltham, USA) using the ERIC primers (ERIC1R: 5'-ATGTAAGCTCCTGGGGATTCAC-3', ERIC2: 5'-AAGTAAGTGACTGGGGTGAGCG-3')[7]. The reaction system was optimized and determined with orthogonal array design and statistic analysis method as previously described[15]. PCR consisted of 2.5 μL 10 × buffer, 200 μmol/L dNTP, 2.5 mmol/L Mg^{2+}, 0.4 μmol/L primer, 1U HotstarTaq DNA polymerase and 2 μL DNA template (or correspondingly 2 μL sterile distilled water in controls) in a total 25 μL volume. PCR conditions were as follows: an initial denaturation at 94℃ for 5 min, followed by 35 cycles of denaturing at 95℃ for 50 s, annealing at 49℃ for 30 s, at 46℃ for 30 s, extension at 72℃ for 3 min, and a final extension at 72℃ for 9 min. PCR products were separated by electrophoresis on 2% agarose gel (Agarose LE, Mdbio, Inc.) containing 0.5 μg/mL ethidium bromide and observed under UV light by Tannon GIS2010 Image System Ver. 3.73 (Tanon, Inc., Shanghai, China). The size and quantity of the amplified fragments were determined using 1 kb plus DNA makers (Tiangen, Inc., Beijing, China).

Statistical analysis of ERIC-PCR fingerprint

ERIC-PCR profiles were analyzed using the Gel Compare function of Tannon GIS2010 Image System Ver. 3.73 and transformed to data sets by taking into account the relative square root of the area under each PCR peak and abundance of each peak. Similarities between samples and their temporal stability were determined by calculating Sorenson's pairwise similarity coefficient (Cs), which is commonly used to compare the species composition of different ecosystems. Two identical profiles create a value of 100%, whereas two completely different profiles result in a value of 0%.

$$Cs\ (\%) = (2 \times j)/(a+b) \times 100\%$$

where 'a' is the number of total bands in the ERIC-PCR pattern for one sample, 'b' is the number for the other, and 'j' is the number of the common bands shared by both samples[16].

Shannon's index (H'), which originally refers to the community richness, was also employed to measure the distribution of PCR bands in our study. We used it to describe the quantitative difference in intestinal microflora under different conditions, although each ERIC-PCR band does not have to stand for one individual bacterial species.

$$H' = -\sum(Pi)(\ln Pi)$$

where P*i* is the relative abundance of each band, calculated as the proportion of the *i*th band in the fringerprint[16-18].

Results were described as mean ± SE. The statistical significance ($P < 0.05$) of difference between means was determined using paired-samples *t* test or ANOVA with SPSS version 11.5 (SPSS Inc., Chicago, USA), where appropriate.

RESULTS

ERIC-PCR fingerprint of intestinal microflora in naive rats

ERIC-PCR profiles of total fecal DNA were obtained for samples collected from naive rats before induction. Repeats of fingerprints showed that there were 9-12 fragments ranging 120-3000 bp with various intensities. There was a considerable variation of ERIC-PCR profiles between individual rats, in which only approximately 50% similarity was seen (Figure 1A). However, samples collected on different days from the same rat showed much a better consistency, with a similarity (Cs) ranging 63%-88% (data not shown). The occurrence of each fragment was calculated using Tannon GIS2010 Image System. Two fragments (590

Table 2 Occurrence and preliminary biomarkers of intestinal microflora ERIC-PCR fingerprinting in rats with Pi-deficiency induced by *Radix* and *Rhizoma Rhei* (n = 100)

Fragments	Occurrence (%)		Net integral area			Abundance		
	Before administration (healthy)	After administration (Pi-deficiency)	Before administration (healthy)	After administration (Pi-deficiency)	Range	Before administration (healthy)	After administration (Pi-deficiency)	Range
590 bp	79	53	851.65 ± 68.00	385.76 ± 37.63[a]	Decrease 55%	18.49 ± 1.36	11.69 ± 1.05[a]	Decrease 37%
380 bp	33	94	98.61 ± 18.09	563.64 ± 32.94[a]	Increase 470%	2.74 ± 0.58	19.16 ± 1.30[a]	Increase 590%
300 bp	75	10	604.93 ± 46.67	70.87 ± 23.12[a]	Decrease 88%	12.70 ± 0.94	2.31 ± 0.71[a]	Decrease 82%

Paired samples t test, [a]$P < 0.05$ *vs* before administration.

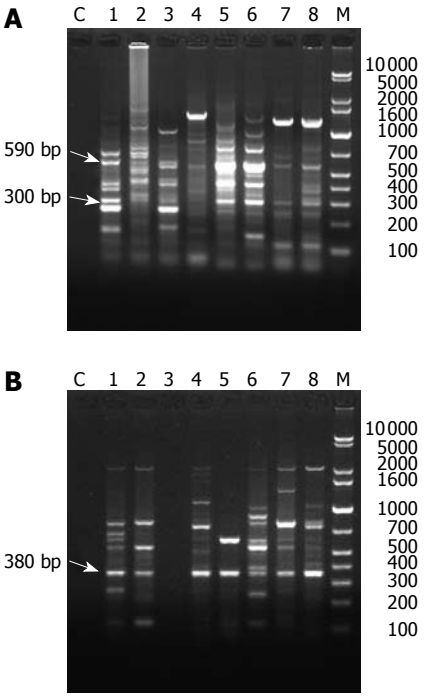

Figure 1 ERIC-PCR fingerprinting of intestinal microflora from feces of 8 out of 128 rats before (A) and after (B) Pi-deficiency induced by *Radix* and *Rhizoma Rhei*. C: Water administration; M: Ladder; lanes 1-8: rats. Rat No. 3 died of trauma after uptake of *Radix* and *Rhizoma Rhei*.

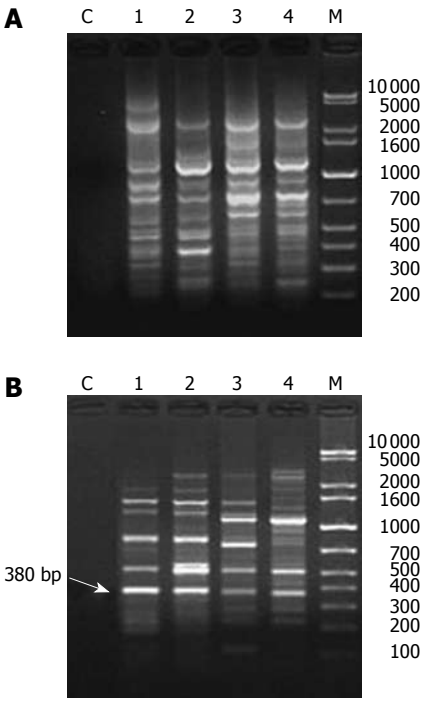

Figure 2 ERIC-PCR fingerprinting of intestinal microflora from feces of 4 out of 128 rats before (A) and after (B) Pi-deficiency induced by Folium Sennae. C: Water administration; M: Ladder; lanes 1-4: rats.

bp and 300 bp) showed a higher occurrence of 83% and 74% respectively (occurrence > 70%) among the fingerprints of 128 rats, indicating that these two predominant bands are likely to be populations-associated naive rat gastrointestine.

ERIC-PCR fingerprint of intestinal microflora in Pi-deficient rats

Symptoms of Pi-deficiency[1-4], including humped back, narrow eyes, watery stools, listlessness, lack of appetite and weight loss[14] occurred in the rats that received *Radix* and *Rhizoma Rhei* or *Folium Sennae*. A remarkable difference in ERIC-PCR profile was found between rats with Pi-deficiency induced by *Radix* and *Rhizoma Rhei* or *Folium Sennae* and normal rats (Figures 1 and 2). Shannon's index (H') of the rat Pi-deficiency model (1.77 ± 0.03, n = 200) was significantly lower than that of normal ones (2.02 ± 0.02, $P < 0.05$, n = 200), indicating that altered profiles and lesser diversities of ERIC-PCR fingerprints are in the status of Pi-deficiency. The similarity (Cs) of ERIC-PCR fingerprints of the same rat before and after Pi-deficiency induction decreased to approximately 39% in groups 2-16, whereas 62% in control group that received distilled water only ($P < 0.05$), suggesting that the constitution of intestinal bacterial community in Pi-deficiency rats is significantly different from that in normal rats.

Molecular markers of intestinal microflora in rats with Pi-deficiency induced by Radix and Rhizoma Rhei

Analysis of ERIC-PCR profiles for 100 rats with Pi-deficiency induced by *Radix* and *Rhizoma Rhei* implied that three fragments (590, 380 and 300 bp) showed that *Radix* and *Rhizoma Rhei* administration can induce significant changes in abundance and band net integral area ($P < 0.05$), as well as the occurrence of those fragments (Table 2). The 590 bp and 300 bp fragments, especially the 300 bp fragment, were shown in most normal rats, but in much fewer rats after Pi-deficiency induction (Figure 1B). Different from the 590 bp and

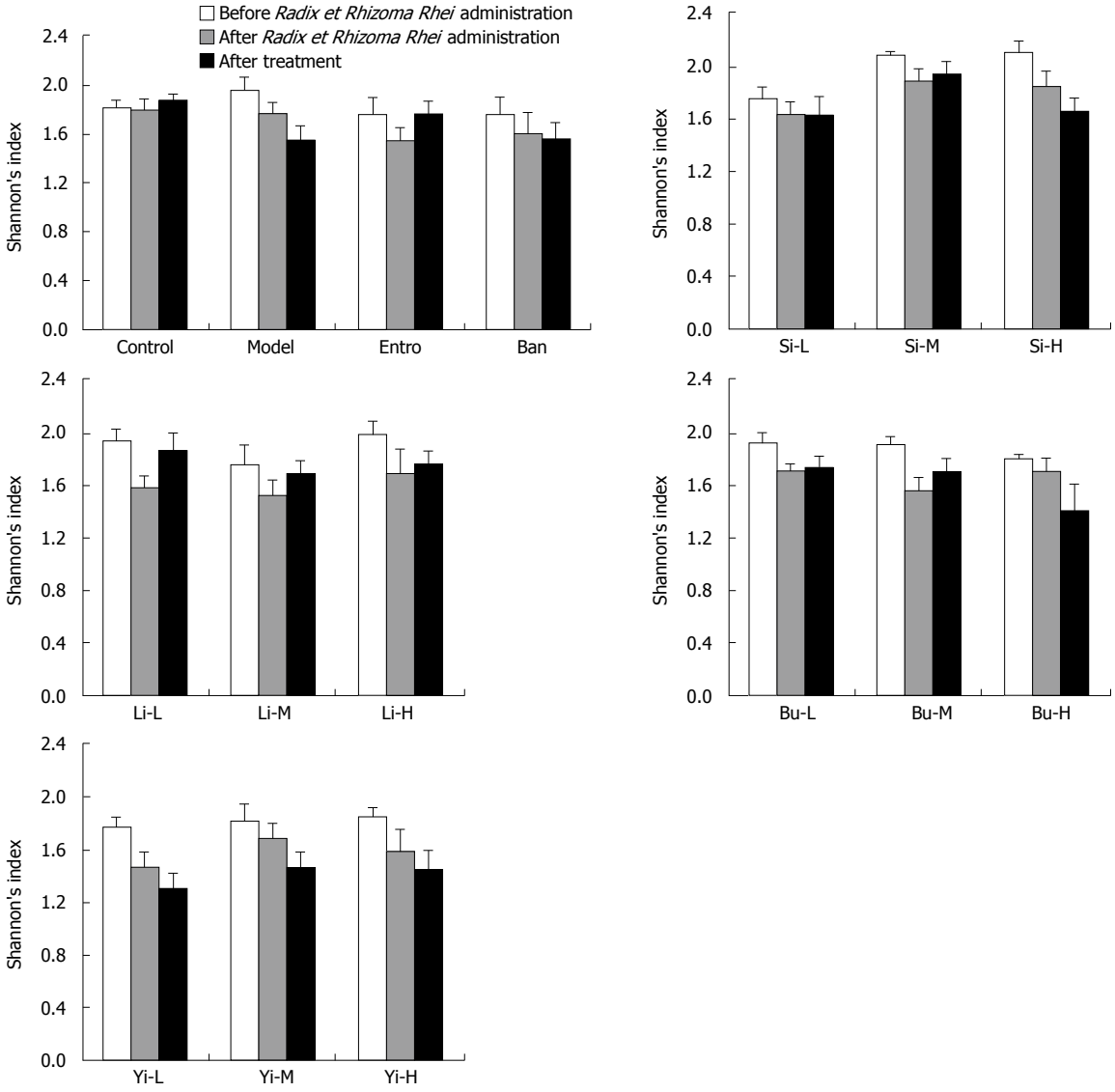

Figure 3 Shannon's index of ERIC-PCR fingerprintings of Pi-deficient rats before and after TCM treatment. Pi-deficiency was induced by *Radix* and *Rhizoma Rhei* first in all groups except for the control group (Group 1) that received distilled water. Control: Group 1 received distilled water in both inducement and treatment phases. Model: Group 2 received *Radix et Rhizoma Rhei* but distilled water during treatment; Entro: Group 3 received Entrocoordinatibiogen during treatment; Ban: Group 4 received decoction of *Ban-xia-hou-pu-tang* during treatment; Si-L, Si-M, Si-H: Groups 5-7 received low, middle and high doses of *Si-jun-zi-tang*, respectively; Li-L, Li-M, Li-H: Groups 8-10 received low, middle and high doses of *Li-zhong-tang*, respectively; Bu-L, Bu-M, Bu-H: Group 11-13 received low, middle and high doses of *Buzhong Yiqi Tang*, respectively; Yi-L, Yi-M, Yi-H: Groups 14-16, received low, middle and high doses of *Yi-wei-tang*, respectively.

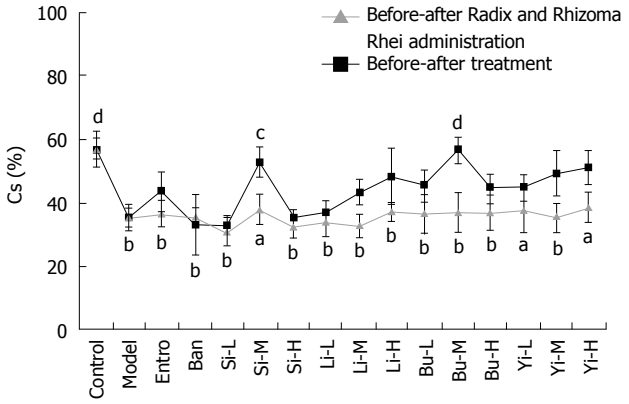

Figure 4 Similarity coefficient (Cs) for ERIC-PCR fingerprintings of Pi-deficient rats before and after treatment. Samples were the same as in Figure 3. Gray: Cs before and after *Radix* and *Rhizoma Rhei* uptake; Black: Cs before and after treatment. Statistical significance of differences was calculated by One-Way ANOVA. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control group; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* model group.

300 bp fragments, the 380 bp fragment was not seen in normal rats, but in most Pi-deficient rats, indicating that *Radix* and *Rhizoma Rhei* administration can induce great changes in the proportion of individual bacterial species. The rest fragments were randomly detected in either normal or Pi-deficient rats, with no correlation between the presence of fragments and Pi-deficiency. Therefore, these fragments (590, 380 and 300 bp) were selected as preliminary biomarkers for intestinal microflora ERIC-PCR fingerprints of rats with Pi-deficiency induced by *Radix* and *Rhizoma Rhei*.

In order to further identify the optimal biomarker of ERIC-PCR fingerprints for rats with Pi-deficiency, the profile of preliminary biomarkers (590, 380 and 300 bp) in different groups of Pi-deficient rats that received TCM treatment was also investigated. As a result, 4 TCM recipes restored the net integral area and abundance of 380 bp fragment to a certain extent. However, changes

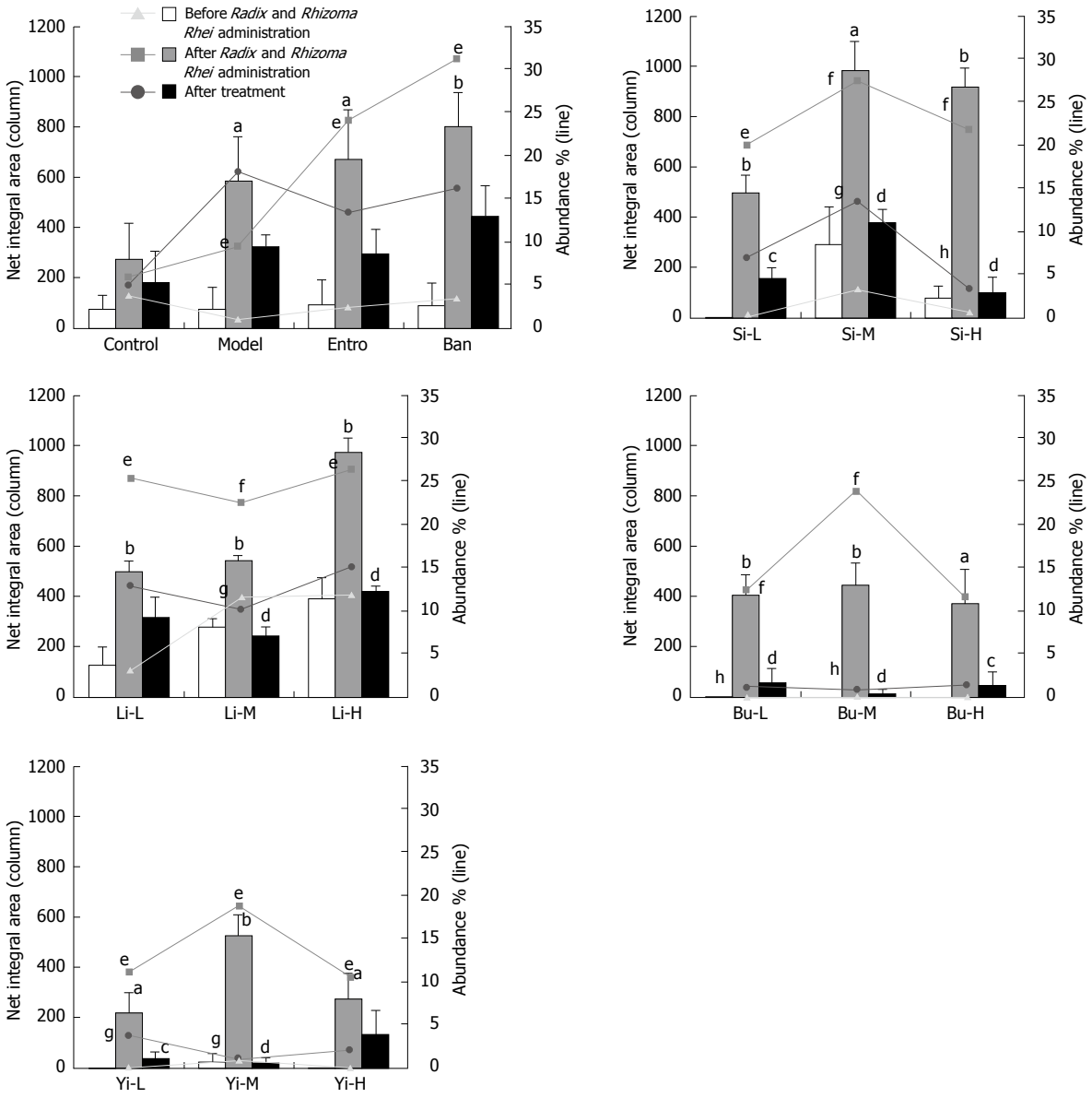

Figure 5 Net integral area and abundance of the 380 bp fragment in rats with Pi-deficiency induced by *Radix* and *Rhizoma Rhei* and after treatment. Columns: Net integral area of the 380 bp fragment; Lines: Abundance of 380 bp fragment. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* healthy condition; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* Pi-deficiency status; $^{e}P < 0.05$, $^{f}P < 0.01$ *vs* healthy condition; $^{g}P < 0.05$, $^{h}P < 0.01$ *vs* Pi-deficiency status. Samples were the same as in Figure 3.

in 590 bp and 300 bp fragments were not as significant as in 380 bp fragment (data not shown). Given the data above, the 380 bp fragment was identified as the biomarker of ERIC-PCR fingerprints for rats with Pi-deficiency induced by *Radix* and *Rhizoma Rhei*, the net integral area and abundance of the 380 bp fragment could therefore be used as parameters to evaluate the therapeutic effects of TCM on Pi-deficiency.

Additionally, in 7 out of 12 Pi-deficient groups that received TCM recipes, The Shannon's index (H') of ERIC-PCR fingerprints was restored (Figure 3). A similar trend of the similarity (Cs) of ERIC-PCR fingerprints was seen in those groups that received TCM recipes (Figure 4), indicating that Shannon's index (H') and Sorenson's pairwise similarity coefficient (Cs) can also be considered as biomarkers for Pi-deficiency and used to evaluate the therapeutic effects of TCM on Pi-deficiency.

Evaluation of therapeutic effects of TCM recipes on P1-deficiency induced by Radix and *Rhizoma Rhei using the identified biomarkers*

As shown in Figures 3-5 and Table 1, Si Junzi Tang reduced the net integral area and abundance of the 380 bp fragment and increased the Sorenson's pairwise similarity coefficient (Cs) in a dose-dependent manner. The effects were most significant at the dose of 3.5 g crude drug/kg. However, the Shannon's index (H') increased after treatment with Si Junzi Tang at the dose of 3.5 g crude drug/kg, but decreased after treatment with Si Junzi Tang at a higher dose of 10.5 g crude drug/kg.

The net integral area and abundance of the 380 bp were significantly different before and after treatment with Lizhong Tang at the dose of 5.4 g. The Sorenson's pairwise similarity coefficient (Cs) increased in a dose-dependent manner and the Shannon's index (H') also increased at the three doses with no significant

difference.

Moreover, Buzhong Yiqi Tang also significantly decreased the 380 bp fragment and increased the Sorenson's pairwise similarity coefficient (Cs) in a dose-dependent manner, in which the maximal and significant effects were shown at the dose of 4.8 g crude drug/kg. Buzhong Yiqi Tang increased and decreased the Shannon's index (H') at the doses of 1.6 and 4.8 g crude drug/kg, and 14.4 g crude drug/kg, respectively.

The net integral area and abundance of the 380 bp were decreased after treatment with Yiwei Tang, indicating that the effect is statistically significant at the dose of 1.9/5.8 g crude drug/kg. Yiwei Tang increased the Sorenson's pairwise similarity coefficient (Cs) in a dose-dependent manner. However, the Shannon's index (H') was lower than that before treatment.

The H' and Cs values as well as the net integral area and abundance of the 380 bp fragment were decreased in rats that received water, which might be due to the long-term Pi-deficiency. A similar trend was seen in the group that received Banxia Houpu Tang. Entrocoordinatibiogen increased the Shannon's index (H') and Sorenson's pairwise similarity coefficient (Cs) ($P < 0.05$), but had no significant effects on the net integral area and abundance of the 380 bp fragment.

Evaluation of therapeutic effects of TCM on Pi-deficieny induced by Folium Sennae

The four biomarkers (H', Cs, net internal area and abundance of the 380 bp fragment) that were identified in rats with Pi-deficiency induced by *Radix* and *Rhizoma Rhei* were also proved to be valid for *Folium Sennae* induced Pi-deficiency. The four recipes (Si Junzi Tang, Lizhong Tang, Buzhong Yiqi Tang and Yiwei Tang) for Pi-deficiency significantly reduced the net integral area and abundance of the 380 bp fragment at smaller and medium doses, but significantly increased the Shannon's index (H') and Sorenson's pairwise similarity coefficient (Cs) (data not shown).

DISCUSSION

This study reported the changes of intestinal microflora in rats with Pi-deficiency induced by *Radix* and *Rhizoma Rhei* or *Folium Sennae*. ERIC-PCR fingerprinting system is highly reproducible when it is used to examine the status of intestinal microflora in rats. In this study, fingerprints of the Sorenson's pairwise similarity coefficient (Cs) from the same DNA extraction were over 95%, respectively.

The dominating intestinal microbial population may vary in subjects due to changed physiological conditions. The replicates (collected on different days) of PCR fingerprints from the same rat showed a high reproducibility (Cs > 75%). However, this value decreased to 57% after water administration, indicating that water administration can affect the intestinal physiology after intragastric operation. These results indicate that ERIC-PCR is a sensitive tool for examining the structure of fecal bacterial community.

Entrocoordinatibiogen (Shenyang No. 1 Pharmaceutical Factory, Shenyang, China) consisting of bacillus licheniformis, a kind of probiotics and a biotherapeutic agent modulating microdysbiosis of intestine, is used in treatment of acute bacillary dysentery. Based on the analysis of ERIC-PCR intestinal microflora molecular markers, the rats that received Entrocoordinatibiogen had a certain extent of recovery as indicated by the increased Shannon's index (H') and Sorenson's pairwise similarity coefficient (Cs). However, neither the net integral area nor the abundance of 380 bp fragment significantly decreased. The 4 major clinical recipes for Pi-deficiency recovered the activities of Pi-deficient rats, especially Si Junzi Tang and Buzhong Yiqi Tang at their medium dose (equivalent to the clinical dose). These results strongly support the rationale behind the current common use of these two recipes for Pi-deficiency[12,14,19]. However, it should be noted that the higher dose of these recipes did not show a better therapeutic effect on Pi-deficiency in the present study. A possible explanation of this phenomenon might be that the recipes have some anti-microorganism actions on Pi-deficiency. It was reported that Si Junzi Tang and some TCM recipes have certain modulating functions in intestinal flora[20-22].

The pathogenesis of Pi-deficiency induced by *Folium Sennae* or by *Radix* and *Rhizoma Rhei* is similar. *Folium Sennae, Radix* and *Rhizoma Rhei*, classified as "bitter-cold" in terms of taste and properties, can simulate the intestinal motility and secretion to induce diarrhea, which results in Pi-deficiency. However, the diarrhea-inducing action of *Folium Sennae* is weaker than that of *Radix* and *Rhizoma Rhei*. That is why the four recipes showed a better recovery profile for *Folium Sennae*-induced Pi-deficiency than that for *Radix* and *Rhizoma Rhei*-induced Pi-deficiency, in the present study.

In conclusion, Pi-deficiency, one of the most common digestive system diseases, is generally caused by the change in intestinal microflora. Although the underlying mechanism of action of TCM is not completely understood, it has been known that TCM has positive effects on some syndromes including Pi-deficiency. ERIC-PCR fingerprints can be used to screen changes in composition of bacterial communities associated with the development of intestinal disease, and to investigate the pharmacodynamic effect of TCM on intestinal microflora or intestinal diseases such as Pi-deficiency.

COMMENTS

Background

Pi-deficiency, a clinical syndrome in traditional Chinese medicine (TCM), is one of the most common digestive system diseases and generally considered to be associated with abnormalities of gastrointestinal microflora.

Research frontiers

Although the underlying mechanism of action of TCM is not completely understood, it has been known that TCM has positive effects on some syndromes including Pi-deficiency. It was reported that some TCM have certain modulating functions in intestinal flora.

Innovations and breakthroughs

In this study, the authors used the molecular markers in study of Pi-deficiency

syndrome, changes in intestinal microflora, and evaluation of the therapeutic effects of traditional Chinese drugs. This is the first study reporting the changes in intestinal microflora of Pi-deficient rats using the enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR) fingerprint profiles.

Applications

ERIC-PCR fingerprints can be used to screen changes in the composition of bacterial communities associated with the development of intestinal disease, and to investigate the pharmacodynamic effect of TCM on intestinal microflora or intestinal diseases including Pi-deficiency.

Terminology

ERIC-PCR is a PCR-based technique in which DNA is isolated from a mixed sample and amplified using conserved ERIC primers targeting short repetitive sequences which are dispersed throughout various bacterial genomes.

Peer review

The authors identified the molecular markers of intestinal microflora by modified ERIC-PCR in rats with Pi-deficiency induced by administration of *Radix* and *Rhizoma Rhei*. In addition, data on the effect of several decoctions on P-deficiency induced by *Radix* and *Rhizoma Rhei* are interesting and seem reliable. The study is interesting and well-designed.

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ORIGINAL ARTICLES

FAT10 level in human gastric cancer and its relation with mutant p53 level, lymph node metastasis and TNM staging

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Abstract

AIM: To investigate the role of FAT10 and mutant p53 in the pathogenesis, severity and prognosis of gastric cancer.

METHODS: FAT10, mutant p53 mRNA and protein levels were measured by reverse transcription (RT)-PCR and immunohistochemistry in gastric cancer tissue (n = 62), tumor-adjacent tissue (n = 62) and normal gastric tissue (n = 62). Relation of FAT10 and mutant p53 expression with clinicopathological features and clinical outcomes of gastric cancer patients were analyzed.

RESULTS: The FAT10, mutant p53 mRNA and protein levels were significantly higher in gastric cancer than in its adjacent and normal tissue. The FAT10 and mutant p53 levels in gastric cancer tissue were significantly correlated with lymph node metastasis and tumor, nodes, metastasis (TNM) staging. Moreover, the high FAT10 level was associated with the overall survival rate of patients. Multivariate Cox-proportional hazards model analysis showed that mRNA and protein levels of FAT10 and mutant p53, lymph node metastasis, distant metastasis and TNM stage were the independent prognostic factors for gastric cancer.

CONCLUSION: FAT10 may be involved in gastric carcinogenesis, and is a potential marker for the prognosis of gastric cancer patients. FAT10 and mutant p53 may play a common role in the carcinogenesis of gastric cancer.

INTRODUCTION

FAT10, also known as diubiquitin, is a ubiquitin-like modifier (UBL) of the ubiquitin protein family, first discovered by Fan *et al*[1] in mapping HLA-F gene in 1996. It has been shown that FAT10 is expressed in mature B cells and dendritic cells[2]. It has been reported that FAT10 regulates cell-cycle and non-covalently binds to the human spindle assembly checkpoint protein (MAD2) that is responsible for the maintenance of spindle integrity during mitosis. Inhibition of MAD2 may lead to chromosomal instability, a common feature of tumorigenesis[3,4]. Lee *et al*[5] found that FAT10 is up-regulated in liver, uterine cervix, ovarian, rectal, pancreatic cancers and small intestinal adenocarcinoma, suggesting that FAT10 plays an important role in tumorigenesis.

P53 gene is located on the short arm of chromosome 17 and classified into wild *p53* and mutant *p53*. P53 protein depletion or gene mutation has been detected in over 50% of all cancers. Under the regulation of upstream signals such as DNA damage, proto-oncogene activity, spindle damage and hypoxia, p53 is activated and functions as a modifier in the processes of cell apoptosis, cell cycle arrest, and DNA repair[6,7]. It was reported that damaged DNA enters into S stage, changes cell hereditary characteristics, and finally induces tumorigenesis when p53 is deleted or mutated[8]. There is evidence that the p53 mutation rate is higher in gastric cancer with atrophic gastritis than in that without atrophic gastritis[9]. Rugge *et al*[10] demonstrated

that the *p53* gene mutation rate is 9.7% in patients (less than 40-year old) with gastric cancer of intestinal type, and lower than in old people (40%-60%) and that the p53 mutation rate is lower in young (6.8%) than in old people (10%-25%) with gastric cancer of diffuse type. Moreover, *Helicobacter pylori* (*H pylori*) infection is an important risk factor for gastric tumorigenesis, whereas patients with gastric cancer and *H pylori*-related cytotoxin-associated gene (CagA) are often accompanied with p53 mutation[11], suggesting that p53 gene mutation also plays an important role in gastric tumorigenesis.

Gastric cancer, one of the most common malignant tumors, is a leading cause of cancer-related death worldwide. The mortality of male and female patients with gastric cancer is on the top of the list in China[12]. Although various genetic and molecular alterations have been found in gastric cancer that underly the malignant transformation of gastric mucosa during the multi-step process of carcinogenesis, the detailed mechanism underlying the development of gastric cancer still remains uncertain. It has recently proposed that FAT10 is a downstream target of p53, and dysregulation of FAT10 expression in p53-defective cells can contribute to carcinogenesis[13]. Therefore, it would be of importance if the function of both FAT10 and p53 and their correlation are investigated in human beings. Furthermore, although many researches are available on the structure and function of FAT10, and its inducing factors, little is known about the role of FAT10 in gastric tumorigenesis and its relation with mutant p53 and other gastric cancer biomarkers. In the present study, we analyzed the expression of FAT10 and mutant p53 in gastric cancer tissue and its adjacent tissue and normal gastric mucosa tissue, in an attempt to discover the potential role of FAT10 in the development of gastric cancer.

MATERIALS AND METHODS

Gastric cancer specimens

In this study, gastric cancer tissue and its adjacent tissue (within 2 cm next to the margin of tumor tissue) and normal gastric tissue (more than 5-10 cm next to the margin of tumor tissue) were obtained from 62 gastric cancer patients who were underwent surgical resection in the First Affiliated Hospital of Medical College, Zhejiang University, from March 2003 to May 2004. None of the patients received any preoperative therapies such as chemotherapy or radiotherapy. The patients consisted of 38 males and 24 females, their age ranged 21- 86 years (mean age: 59.62 years). The tumor, nodes, metastasis (TNM) stage of gastric cancer referring to the p-TNM stage were promulgated by the International Union against Cancer (UICC) in 1997. This study was approved by the Hospital Review Board and written consent was obtained from each involved patient.

Immunohistochemistry

FAT10 and mutant p53 protein levels were routinely measured by immuno-histochemistry. Briefly, gastric cancer tissue and its adjacent tissue and normal gastric tissue were sequentially fixed with 10% formalin, embedded in paraffin and cut into 4-μm thick sections. The sections were deparaffinaged and endogenous peroxidase was blocked with H_2O_2. Antigen retrieval was performed by heating the sections in a 0.01 mol/L citrate buffer in a microwave oven. Nonspecific binding was blocked by incubating the sections with normal rabbit serum for 20 min. The sections were then incubated at 37℃ for 2 h with either polyclonal FAT10 antibody (Shanghai Jintai Biological Science and Technology Ltd, China) or monoclonal mutant P53 antibody (Beijing Zhongshan Biological Science and Technology Ltd, China). Controls without primary antibodies were also included. After washed three times with PBS, the sections were incubated with biotin-conjugated secondary antibody (Shanghai Jintai Biological Science and Technology Ltd, China) for 40 min at room temperature. Immunocomplexes were detected with 3, 3-diaminobenzidine (Fuzhou Maixin Biological Science and Technology Ltd, China) that acts as a chromogen and results in deposition of brown reaction products. Species with 0%, about 10%, about 50% of positively stained cells were scored as -, +, and ++, respectively.

RNA extraction and reverse transcription polymerase chain reaction (RT-PCR)

RNA was extracted using Trizol reagent with one-step extraction method. In brief, 1 mL Trizol reagent was added to cultured cells or to approximately 100 mg tissue specimens, respectively, and total RNA was isolated following the manufacturer's instructions. RNA integrity was assessed by agarose gel electrophoresis when clear 18 S and 28 S strips appeared on the gel, whereas total RNA concentration was measured with a spectrophotometer ($A_{260/280}$ Ratios of 1.8-2.0) following its manufacturer's instructions. Total RNA (5 μL) was reversed into cDNA in a 24 μL reaction system at 42℃ for 60 min and at 70℃ for 10 min, sequentially. The mixture contained 5 μL 5-RT-buffer, 1 μL Oligo (dT), 2 μL 10 mmol/L dNTPs mix, 1 μL ribonuclease inhibitor (20 U/μL), 1 μL M-MMLV reverse transcriptase (200 U/μL), and 9 μL DEPC water. The final RT reaction solution was used in PCR (GeneAmp 2400 PCR System, Perkin Elmer Company, USA). The primers and lengths of PCR amplifications are listed in Table 1. DNA products were run in agarose gel at 80-100 V and then analyzed by BIO-RAD image acquisition with an analysis system, followed by semi-quantitative analysis with Quantity One software. The 295 bp, 478 bp, and 492 bp long fragments were detected under UV light. Gray scale scanning was performed on electrophoresis strips using an image acquisition and analysis system.

Statistical analysis

Immunohistochemical expression was defined as positive when moderate to strong nuclear staining was observed in more than 10% cells. χ^2 test was used to determine

Table 1 Primers used in this study

Designation	Sequences	PCR products (bp)
β-actin		
Upstream primer	5'-TCACCCACACCGT-GCCCATCTACGA-3'	295
Downstream primer	5'-CAGCGGAACCGCT-CATTGCCAACGG-3'	
FAT10		
Upstream primer	5'-AATGCTTCCTGCCTCT-GTGT-3'	478
Downstream primer	5'-GCCGTAATCTGCCAT-CATCT-3'	
Mutant p53		
Upstream primer	5'-CCTATGGAAAC-TACTTCCTGAAAACAA-3'	492
Downstream primer	5'-ACAGCATCAAAT-CATCCATTGC-3′	

the difference in FAT10 and p53 protein levels between different groups and the correlation between positive FAT10 ratio and clinicopathological parameters of patients. RT-PCR semi-quantitative results were expressed as mean ± SD. Student's *t*-test was employed to analyze differences in mRNA levels. Relation between FAT10 and p53 was statistically analyzed using Spearman's rank correlation. Overall survival rate was calculated by Kaplan-Meier curves. Cox proportional hazards model was used to examine the effect of potential prognostic variables on survival. Statistical analysis was performed with SPSS software version 12.0. $P < 0.05$ was considered statistically significant.

RESULTS

Detection of FAT10 and mutant p53 protein in different gastric tissues by immunohistochemistry

FAT10 protein was mainly detected in nuclei of malignant and benign cells which were stained brown-yellow (Figure 1A). Positive staining of FAT10 cells was found in tumor tissue and its adjacent tissue and normal tissue samples from 32 (51.61%), 8 (12.90%) and 4 (6.45%) of the 62 cases. The FAT10 level was significantly different in gastric cancer tissue, and its adjacent tissue and normal tissue ($\chi^2 = 40.96$, $P < 0.01$). The rate of positive FAT10 cells in gastric cancer tissue was significantly higher than that in its adjacent tissue and normal tissue ($P < 0.01$) but was not significantly different between adjacent tissue and normal tissue (Table 2). Mutant P53 was expressed mainly in nuclei and cytoplasm (Figure 1B). Positive immunostaining of mutant fp53 was noted in 45.16% (28/62) of tumor tissue samples, 14.51% (9/62) of adjacent tissue samples and 9.63% (6/62) of normal tissue samples ($\chi^2 = 25.83$, $P < 0.01$). The positive rate of mutant p53 expression in tumor tissue was significantly higher than that in adjacent tissue and normal tissue ($P < 0.01$, Table 2).

Detection of FAT10 and mutant p53 mRNA in different gastric tissues by RT-PCR

The levels of FAT10 and mutant p53-mRNA in tumor tissue and non-tumor tissue were measured. RT-PCR analysis revealed that the relative FAT10-mRNA expression in gastric cancer tissue was significantly higher than that in its adjacent tissue ($t = 3.12$, $P < 0.01$) and normal tissue ($t = 4.64$, $P < 0.01$), whereas no significant difference was detected between tumor-adjacent and normal tissues ($t = 1.03$, Figure 2). Mutant P53-mRNA expression was significantly higher in gastric cancer tissue than in its adjacent tissue ($t = 6.79$, $P < 0.01$) and normal tissue ($t = 5.51$, $P < 0.01$). The difference in mutant p53-mRNA expression between adjacent and normal tissue was not statistically significant ($t = 1.22$, Figure 2).

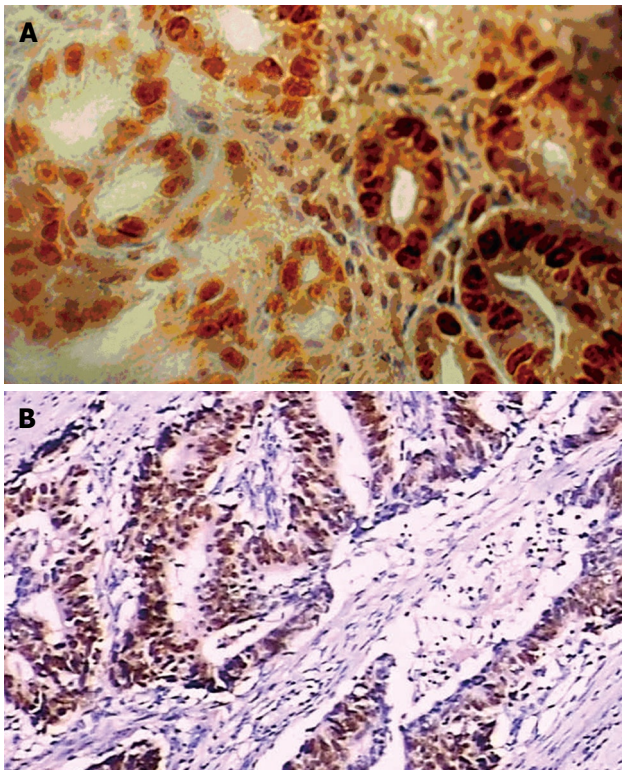

Figure 1 Expression of FAT10 (A) and mutant p53 (B) in gastric cancer tissue (× 400).

Table 2 FAT10 and mutant p53 protein expression in gastric cancer tissue and its adjacent tissue and normal tissue *n* (%)

Tissue	FAT10		Mutant p53	
	Negative	Positive	Negative	Positive
Gastric tissue	30	32 (51.61)	34	28 (45.16)
Tumor-adjacent tissue	54	8 (12.90)	53	9 (14.51)
Normal gastric tissue	58	4 (6.45)	56	6 (9.68)

Relation between FAT10 protein and mRNA levels and clinicopathological features of gastric cancer

To test the potential value of FAT10 as a gastric cancer biomarker, we performed χ^2 test to evaluate the correlation of FAT10 expression with clinicopathological features of gastric cancer (Table 3). The positive rate of FAT10 expression in gastric cancer with regional lymph node metastasis was significantly higher than that without

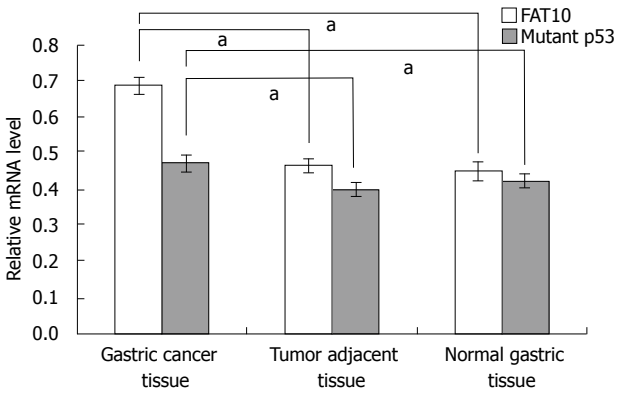

Figure 2 Relative mRNA levels of FAT10 and mutant p53 in different tissues. The column represents the relative gray values of FAT10 and mutant p53 mRNA by normalizing the gray value of β-actin. $^aP < 0.05$ (for FAT10: 0.689 ± 0.023 in gastric cancer tissue, 0.463 ± 0.019 in tumor adjacent tissue, 0.451 ± 0.028 in normal gastric tissue; for mutant p53: 0.471 ± 0.021 in gastric cancer tissue, 0.398 ± 0.017 in tumor adjacent tissue, 0.421 ± 0.019 in normal gastric tissue).

Table 3 Relation between FAT10 expression and clinicopathologic factors for gastric cancer

Clinic pathologic factors	Samples	FAT10 positive samples (%)	χ^2	*P*
Age (yr)				
< 50	19	7 (36.84)	2.39	> 0.05
⩾ 50	43	25 (58.14)		
Gender				
Male	38	19 (50.00)	0.1	> 0.05
Female	24	13 (54.16)		
Tumor size (cm)				
< 5	33	16 (48.48)	0.28	> 0.05
⩾ 5	29	16 (55.17)		
Location				
Antrum	31	15 (48.39)	2.81	> 0.05
Angle	5	2 (40.00)		
Body	14	9 (64.29)		
Fundus	4	3 (75.00)		
Cardia	8	3 (37.50)		
Progression degree				
Early stage	11	6 (54.54)	0.05	> 0.05
Progressive stage	51	26 (50.98)		
Differentiation degree				
High, middle	15	7 (46.67)	0.19	> 0.05
Low, none	47	25 (53.19)		
Lymph metastasis				
Positive	39	25 (64.10)	6.57	< 0.05
Negative	23	7 (30.43)		
Distant metastasis				
Positive	15	11 (73.33)	3.74	> 0.05
Negative	47	21 (44.68)		
TNM Staging				
Ⅰ + Ⅱ	21	7 (33.33)	4.25	< 0.05
Ⅲ + Ⅳ	41	25 (60.98)		

regional lymph node metastasis ($P < 0.05$). Furthermore, high FAT10 expression levels were associated with advanced TNM staging (Ⅲ + Ⅳ/ Ⅰ + Ⅱ) ($P < 0.05$). However, there was no significant difference in FAT10 expression, age and gender of patients, tumor size, location, histological grade, and distant metastasis. The expression of FAT10-mRNA was correlated with lymph node status and TNM stage (Ⅲ + Ⅳ/ Ⅰ + Ⅱ)

Table 4 Relation between FAT10-mRNA level and clinicopathological factors for gastric cancer

Clinicopathological factors	Samples (*n*)	Positive samples	Strap gray value (mean ± SD)	*t*-value	*P*
Age (yr)					
< 50	19	10	0.583 ± 0.036	1.12	> 0.05
⩾ 50	43	26	0.611 ± 0.026		
Sex					
Male	38	21	0.622 ± 0.013	1.36	> 0.05
Female	24	15	0.574 ± 0.024		
Tumor size (cm)					
< 5 cm	33	19	0.594 ± 0.022	0.65	> 0.05
⩾ 5 cm	29	17	0.590 ± 0.018		
Location					
Tantrum	31	17	0.537 ± 0.019	1.54	> 0.05
Angle	5	3	0.672 ± 0.037		
Body	14	9	0.594 ± 0.021		
Fundus	4	4	0.611 ± 0.025		
Cardia	8	3	0.529 ± 0.032		
Progression degree					
Early	11	8	0.623 ± 0.023	1.15	> 0.05
Advanced	51	28	0.597 ± 0.020		
Differentiation degree					
Well-middle	15	9	0.564 ± 0.031	1.39	> 0.05
Low-non	47	27	0.595 ± 0.019		
Lymph node metastasis					
Positive	39	27	0.656 ± 0.016	3.37	< 0.01
Negative	23	9	0.531 ± 0.026		
Distant metastasis					
Positive	15	12	0.623 ± 0.033	1.74	> 0.05
Negative	47	24	0.598 ± 0.017		
TNM stage					
Ⅰ + Ⅱ	21	8	0.667 ± 0.023	2.25	< 0.05
Ⅲ + Ⅳ	41	28	0.558 ± 0.015		

Table 5 Correlation between FAT10 and mutant p53 expressions in gastric cancer tissue

p53	Samples	FAT10 expression	
		Positive	Negative
Positive	28	23	5
Negative	34	9	25
Total	62	32	30

($P < 0.05$), but not with age and sex of patients, tumor size, location, histological grade, and distant metastasis, which was similar to the immunohistochemical results (Table 4).

Correlation between FAT10 and mutant p53 in gastric cancer tissue

The expression rate of FAT10 was 82.14% (23/28) and 26.47% (9/34) in positive and negative mutant p53 tumor tissues, respectively (Table 5). The correlation between FAT10 and mutant p53 was analyzed by Spearman rank correlation. The expression of FAT10 was positively correlated with mutant p53 in gastric cancer ($r = 0.865$, $P < 0.05$). Furthermore, Spearman rank correlation analysis showed that FAT10-mRNA was significantly correlated with mutant p53-mRNA ($r = 0.548$, $P < 0.05$).

Predictive role of FAT10 in gastric cancer

To investigate the impact of FAT10 over-expression

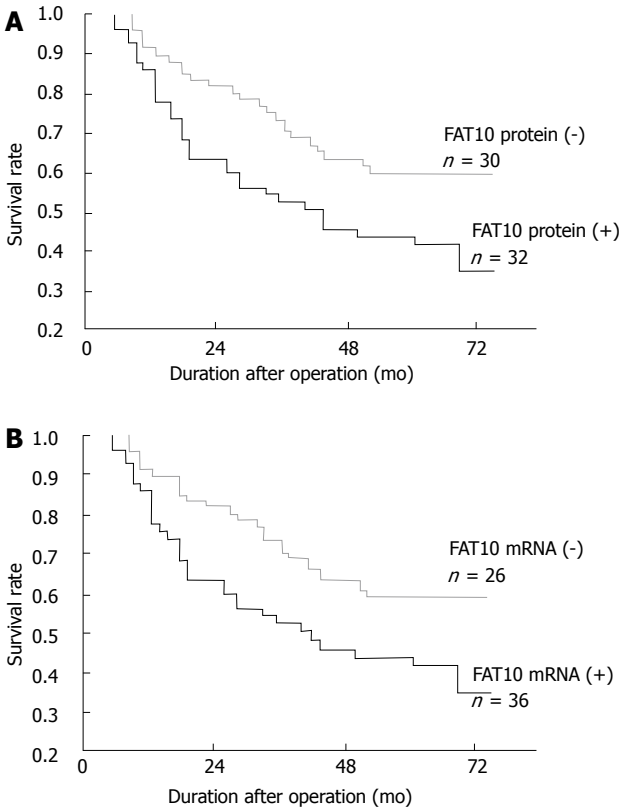

Figure 3 Effect of FAT10 protein (A) and mRNA (B) on the survival rate of gastric cancer patients (Kaplan-Meier survival curve).

Table 6 Cox regression model analysis results of the prognostic factors for gastric cancer

Harzard factors	B	S χ	Wald	υ	Sig.	Exp (B)
Age	0.134	0.144	0.853	1	0.533	1.012
Tumor size	0.243	0.246	0.804	1	0.368	0.763
Location	0.072	0.055	1.846	1	0.173	0.954
Progression	0.144	0.267	0.26	1	0.615	1.148
Differentiation degree	0.544	0.23	2.613	1	0.118	1.223
Lymph node metastasis	0.734	0.384	4.985	1	0.027	1.886
Distant metastasis	0.764	0.326	6.785	1	0.001	1.806
TNM stage	0.698	0.285	7.205	1	0.007	1.745
FAT10 protein	0.661	0.228	8.448	1	0.004	1.516
FAT10 mRNA	0.793	0.245	7.658	1	0.003	1.854
p53 protein	0.669	0.239	6.479	1	0.006	1.611
p53 mRNA	0.758	0.346	6.857	1	0.004	1.521

on the clinical outcome of patients, univariate survival probability curves were plotted with respect to the immunohistochemical and RT-PCR results. Except for 4 patients (2 with positive FAT10 protein and FAT10-mRNA, and 2 with negative FAT10 protein and FAT10-mRNA), the other patients were followed up for 48-72 mo. We found that high FAT10 protein and mRNA levels in gastric cancer showed a tendency towards unfavorable prognosis regarding the overall survival rate as shown by Kaplan-Meier analysis ($P < 0.05$, Figure 3). In the Cox regression model, multivariate survival analyses showed that FAT10 protein and mRNA, as well as lymph node metastasis, distant metastasis, TNM stage, and mutant p53 mRNA protein, were the independent adverse prognostic factors for the overall survival rate (Table 6).

DISCUSSION

FAT10 is a member of the ubiquitin-like modifier family of proteins. Over-expression of the FAT10 gene has been observed in several epithelial cancers and high FAT10 expression can increase chromosome instability by reducing kinetochore localization of MAD2 during the prometaphase stage of cell-cycle[14]. In the present study, we measured the FAT10 protein and mRNA levels in gastric cancer tissue, its adjacent tissue and normal tissue from 62 patients. The immunohistochemical analysis suggested that FAT10 protein was mainly expressed in cell nuclei, indicating that FAT10 may participate in cell cycle regulation. The positive expression rate of FAT10 protein and mRNA in gastric cancer tissue was significantly higher than that in its adjacent tissue and normal tissue, suggesting that FAT10 may play an important role in the process of gastric carcinogenesis

Recent researches have shown that the expression of mutant p53 and FAT10 mRNAs is increased in cancer cell line[15,16]. However, the correlation between mutant p53 and FAT10 has not been analyzed in human gastric cancer. In this study, FAT10 and mutant p53 protein/mRNA were over-expressed in gastric cancer tissue whereas high FAT10 and mutant p53 expression levels in tumor tissue were positively correlated. It was reported that p53 negatively regulates the expression of FAT10 and p53 depletion, thus contributing to tumorigenesis by uncontrolled up-regulation of FAT10[13], suggesting that mutant p53 may also activate the FAT10 gene and promote gastric tumorigenesis due to the loss of its anti-carcinoma effect. Moreover, proinflammatory cytokines up-regulate FAT10 in liver and colon cancer, indicating that they play a potential role in activating FAT10 in gastric tumor and merit further investigation[16]. Although p53 binds to the 5' half consensus sequence of p53-binding site at the FAT10 promoter[13], the exact p53-binding site is still unclear, thus further study is needed.

No report is available at present on the correlation between FAT10 protein expression and clinicopathological characteristics of gastric cancer patients. In the present study, FAT10 protein and mRNA levels were closely correlated with lymph node metastasis and TNM stage (Ⅲ + Ⅳ/ Ⅰ + Ⅱ) ($P < 0.05$), indicating that FAT10 can promote tumor invasion and metastasis, and may be a candidate prognostic factor for lymph node metastasis and tumor progression. Large scale studies are needed to further confirm our findings.

Tumor metastasis has become one of the most challenging problems in tumor therapy. Many efforts have been made to predict gastric cancer behaviors, but specific predictive markers for metastasis and recurrence are still lacking[17]. In our study, the patients with a high FAT10 expression level showed a tendency towards unfavorable prognosis. Since FAT10 and mutant p53 protein and mRNA, lymph node

metastasis, distant metastasis, and TNM stage are the independent prognostic factors for poor patient survival, determination of FAT10 status may be an important step in formulating right therapeutic strategies. Moreover, FAT10 may be related with other predictive biomarkers of tumor metastasis, such as CD44v6, nm23, MTA1 and MMPs.

In conclusion, FAT10 is over-expressed in gastric cancer tissue, and positively correlated with mutant p53 expression, lymph node metastasis and tumor progression, and can promote tumor invasion. FAT10 is of prognostic value for human gastric cancer and is a potential target for cancer biotherapy.

COMMENTS

Background

FAT10 belongs to the ubiquitin-like modifiers of ubiquitin protein family, first discovered in mapping HLA-F gene in 1996, and is expressed in mature B cells and dendritic cells. It has been reported that FAT10 can regulate cell-cycle and may play an important role in tumorigenesis. *P53* gene, located on the short arm of chromosome 17, can be divided into wild *p53* and mutant *p53*. P53 protein depletion or gene mutation has been detected in over 50% of all cancers, suggesting that P53 may play an important role in gastric tumorigenesis. Gastric cancer, one of the most common malignant tumors, is a leading cause of cancer-related death worldwide, and is on the top of the list in China. The detailed mechanism underlying the development of gastric cancer still remains uncertain.

Research frontiers

It has been recently found that FAT10 can non-covalently bind to the human spindle assembly checkpoint protein (MAD2) that is responsible for the maintenance of spindle integrity during mitosis. Inhibition of MAD2 may lead to chromosomal instability, a common feature of tumorigenesis. It has been shown that damaged DNA enters into S stage, changes cell hereditary characteristics, and finally induces tumorigenesis when p53 is deleted or mutated. Moreover, there is evidence that the p53 mutation rate is higher in gastric cancer with atrophic gastritis than in gastric cancer without atrophic gastritis. FAT10 is a downstream target of p53, and down-regulation of FAT10 expression in p53-defective cells contributes to carcinogenesis.

Innovations and breakthroughs

In this study, the FAT10 and mutant p53 mRNA and protein levels were significantly higher in gastric cancer tissue than in its adjacent tissue and normal tissue. The FAT10 and mutant p53 levels in gastric cancer tissue were significantly correlated with lymph node metastasis and TNM staging. Moreover, mRNA and protein levels of FAT10 and mutant p53, lymph node metastasis, distant metastasis, and TNM stage were found to be independent prognostic factors for patients with gastric cancer.

Applications

FAT10 may be a potential marker of gastric cancer prognosis, which needs to be further verified. FAT10 is positively correlated with mutant p53, indicating that it may play a role in carcinogenesis and becomes a novel therapeutic target of gastric cancer.

Terminology

FAT10: a protein belonging to ubiquitin-like modifier (UBL) of ubiquitin protein family, is mainly expressed in mature B cells and dendritic cells and regulates cell-cycle and chromosomal instability, thus playing an important role in tumorigenesis.

Peer review

This study describes the increased FAT10 and mutant p53 mRNA and protein levels in gastric cancer and the correlation between FAT10 and mutant p53. Furthermore, the authors also found that mRNA and protein levels of FAT10 were independent prognostic factors for patients with gastric cancer. These results are innovative, showing that FAT10 may be involved in gastric carcinogenesis and in gastric cancer prognosis. However, further study is needed to further verify their findings.

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ORIGINAL ARTICLES

Induction of apoptosis in human liver carcinoma HepG2 cell line by 5-allyl-7-gen-difluoromethylenechrysin

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Abstract

AIM: To investigate the effect of 5-allyl-7-gen-difluoromethylenechrysin (ADFMChR) on apoptosis of human liver carcinoma HepG2 cell line and the molecular mechanisms involved.

METHODS: HepG2 cells and L-02 cells were cultured *in vitro* and the inhibitory effect of ADFMChR on their proliferation was measured by MTT assay. The apoptosis of HepG2 cells was determined by flow cytometry (FCM) using propidium iodide (PI) fluorescence staining. DNA ladder bands were observed by DNA agarose gel electrophoresis. The influence of ADFMChR on the proxisome proliferator-activated receptor γ (PPARγ), NF-κB, Bcl-2 and Bax protein expression of HepG2 cells were analyzed by Western blotting.

RESULTS: MTT assay showed that ADFMChR significantly inhibited proliferation of HepG2 cells in a dose-dependent manner, with little effect on growth of L-02 cells, and when IC_{50} was measured as 8.45 μmol/L and 191.55 μmol/L respectively, the potency of ADFMChR to HepG2 cells, was found to be similar to 5-fluorouracil (5-FU, IC_{50} was 9.27 μmol/L). The selective index of ADFMChR cytotoxicity to HepG2 cells was 22.67 (191.55/8.45), higher than 5-FU (SI was 7.05 (65.37/9.27). FCM with PI staining demonstrated that the apoptosis rates of HepG2 cells treated with 3.0, 10.0 and 30.0 μmol/L ADFMChR for 48 h were 5.79%, 9.29% and 37.8%, respectively, and were significantly higher when treated with 30.0 μmol/L ADFMChR than when treated with 30.0 μmol/L ChR (16.0%) ($P < 0.05$) and were similar to those obtained with 30.0 μmol/L 5-FU (41.0%). DNA agarose gel electrophoresis showed that treatment of HepG2 cells with 10.0 μmol/L ADFMChR for 48 h and 72 h resulted in typical DNA ladders which could be reversed by 10.00 μmol/L GW9662, a blocker of PPARγ. Western blotting analysis revealed that after 24 h of treatment with 3.0, 10.0, 30.0 μmol/L ADFMChR, PPARγ and Bax protein expression in HepG2 cells increased but Bcl-2 and NF-κB expression decreased; however, pre-incubation with 10.0 μmol/L GW9662 could efficiently antagonize and weaken the regulatory effect of 3.0, 30.0 μmol/L ADFMChR on PPARγ and NF-κB protein expression in HepG2 cells.

CONCLUSION: ADFMChR induces apoptosis of HepG2 cell lines by activating PPARγ, inhibiting protein expression of Bcl-2 and NF-κB, and increasing Bax expression.

Key words: Liver neoplasm; Chrysin; 5-allyl-7-gen-difluoromethylenechrysin; Apoptosis; Proxisome proliferator-activated receptor γ

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Tan XW, Xia H, Xu JH, Cao JG. Induction of apoptosis in human liver carcinoma HepG2 cell line by 5-allyl-7-gen-difluoromethylenechrysin. *World J Gastroenterol* 2009; 15(18): 2234-2239 Available from: URL: http://www.wjgnet.com/1007-9327/15/2234.asp DOI: http://dx.doi.org/10.3748/wjg.15.2234

INTRODUCTION

Human liver carcinoma is the fifth most common cancer in the world and is responsible for > 600 000 deaths

annually[1]. The majority of patients with hepatocellular carcinoma die within 1 year after the diagnosis. At present, the treatment of hepatocellular carcinoma mainly includes surgery and chemotherapy, but the curative effects of the existing chemotherapeutic drugs are not good enough and they have numerous side effects. Therefore, searching for highly efficient antitumor drugs remains a hot research area.

Peroxisome proliferator-activated receptor γ (PPARγ) is a member of the nuclear hormone receptor superfamily; a ligand-dependent transcription factor that plays an important role in lipid and glucose metabolism[2,3]. In recent years, over-expression of PPARγ has been found in a variety of tumor cells and PPARγ agonists can induce apoptosis[4,5]. It has been reported that chrysin (ChR) and its derivatives activate PPARγ to inhibit COX-2 and iNOS activity through various pathways distinguished from thiazolidones[6].

Chrysin (5,7-dihydroxy flavone, ChR) is a kind of flavonoid with pharmacological activities and is widely distributed in the plant kingdom. It has been demonstrated that ChR can markedly inhibit the growth of human thyroid cancer cells[7], and has an effect on the inhibition of proliferation and induction of apoptosis in human myeloid leukemia cells as well[8,9]. Comte *et al*[10] reported that, through alkylation, the hydrophobicity of ChR is increased, its KD value decreased, and its binding affinity towards P-glucoprotein (P-gp) enhanced. We confirmed that a series of B-ring trifluoromethylated derivatives of ChR markedly inhibited the growth of HT-29 and SGC-7901 cell lines[11] and that 5, 7-dihydroxy-8-nitrochrysin (NOChR) had an inhibitory effect on subcutaneously transplanted primary Lewis lung carcinoma in mouse and its spontaneous metastasis in a dose-dependent manner[12]. Our previous study showed that the suppressive effect of 5-allyl-7-gen-difluoromethylenechrysin (ADFMChR) on proliferation of the CoC1 cell line was stronger than that of ChR[13]. However, whether ADFMChR has antitumor effects on human liver carcinoma is unknown.

In this study, we aimed to investigate whether ADFMChR induces apoptosis of HepG2 cell line by activation of PPARγ and whether NF-κB, Bcl-2 and Bax are involved in this mechanism, thereby providing a new opportunity for research with regard to the pharmaceutical prevention and cure of human liver cancer.

MATERIALS AND METHODS

Cell lines and cell culture

HepG2 cells and L-02 cells were purchased from the China Center for Type Culture Collection (CCTCC) and were cultured in RPMI-1640 medium with 10% fetal bovine serum. Antibiotics added were 100 units/mL penicillin and 100 μg/mL streptomycin (Life Technologies, Inc) at 37℃ in a 5% CO_2 incubator.

Medicines and chemical reagents

ADFMChR was synthesized in the Medical College, Hunan Normal University as previously described[14]; with a molecular weight of 344 ku, characteristic yellow crystals and purity of 99.0%, its molecular formula is $C_{19}H_{14}O_4F_2$. ADFMChR was dissolved in dimethyl sulfoxide (DMSO), diluted with phosphate buffer solution (PBS), and finally prepared as 2 mmol/L storage solution after filtration sterilization. RPMI-1640, ChR, MTT and DMSO were purchased from Sigma Company. 5-fluorouracil (5-FU) was from Jinghua Pharmaceutical Corporation Ltd, Nantong. Ladder Apoptotic DNA Ladder Detection Kit was purchased from Bodataike Company, Beijing. Mouse anti-human Bcl-2 monoclonal antibody, mouse anti-human NF-κB monoclonal antibody, mouse anti-human Bax monoclonal antibody and rabbit anti-human PPARγ polyclonal antibody were purchased from Santa Cruz Biotechnology, Inc (U.S.A).

MTT assay

HepG2 cells or L-02 cells were seeded in a 96-well plate at a density of 1.0×10^4 cells/well as previously described[15]. Drugs of different concentrations were added to each well and cultured for 48 h, followed by incubation with 5 mg/L MTT for 4 h. The supernatant was removed after centrifugation. Finally, 100 μL of DMSO was added and absorbance at 490 nm wavelength (A_{490}) was measured by means of Enzyme-labeling instrument (EX-800 type). Relative cell proliferation inhibition rate (IR) = (1 - average A_{490} of the experimental group/average A_{490} of the control group) × 100%.

Flow cytometry (FCM) with propidium iodide (PI) staining

HepG2 cells were treated with serum-free medium for 24 h, followed by treatment with media containing 3.0, 10.0, 30.0 μmol/L ADFMChR, 30.0 μmol/L ChR and 30.0 μmol/L 5-FU for 48 h, respectively. Cells were collected and prepared as a single cell suspension by mechanical blowing with PBS, washed with cold PBS twice, fixed with 700 mL/L alcohol at 4℃ for 24 h, stained with PI and cell apoptosis was detected using FCM (American BD Company, FACS420).

DNA agarose gel electrophoresis

As previously described[16], cells were cultured with 10.0 μmol/L ADFMChR and 10.0 μmol/L ADFMChR plus 10.0 μmol/L GW9662, a PPARγ antagonist, for 0, 24, 48 and 72 h, respectively. Cells were washed twice with PBS and DNA was extracted with an Apoptotic DNA Ladder Detection Kit according to the manufacturer's instructions. The extracted DNA was kept at 4℃ overnight. Then 8.5 μL of DNA sample was mixed with 1.5 μL of 6 × Buffer solution, electrophoresed on 20.0 g/L agarose gel containing ethidium bromide at 40 V, and observed through DBT-08 gel image analysis system.

Western blotting analysis

As previously described[17], cells were treated with 3.0, 10.0, 30.0 μmol/L ADFMChR and 30.0 μmol/L ChR for 24 h, respectively. Cells were collected, washed three times with PBS, lysed in cell lysis buffer containing 0.1 mol/L NaCl, 0.01 mol/L Tris-Cl (pH 7.6), 0.001 mol/L

EDTA (pH 8.0), 1 μg/mL Aprotinin, 100 μg/mL PMSF, and then centrifuged at 13000 × *g* for 10 min at 4℃. The extracted protein sample (25 μg total protein/lane) was added in the same volume of sample buffer and subjected to denaturation at 100℃ for 10 min, then electrophoresed on 100 g/L or 60 g/L SDS-PAGE at 100 mA for 3 h, and finally transferred onto PVDF membrane. The PVDF membrane was treated with TBST containing 50 g/L skimmed milk at room temperature for 2 h, followed by incubation with the primary antibodies PPARγ, NF-κB, Bcl-2 and Bax (1:500 dilution), respectively, at 37℃ for 2 h or at 4℃ overnight. After being washed with TBST for 30 min, the corresponding secondary antibody was added and incubated at room temperature for 1 h. The membrane was then washed three times for 15 min each with TBST. Fluorescence was visualized with enhanced chemiluminescence (Amersham, Arlington Heights, IL). The results were analyzed with Image analyzer and the product of area and optical density was expressed as integral absorbance (IA).

Statistical analysis

Experimental data in each group were presented as mean ± SD. Analysis of variance was performed with SPSS software for windows 15.0 by using one way ANOVA and pairwise comparison with Student's *t* test. $P < 0.05$ was considered statistically significant.

RESULTS

Determination of proliferation of HepG2 and L-02 cell lines by MTT assay

MTT assay showed that ADFMChR markedly inhibited proliferation of HepG2 cells in a dose-dependent manner (Figure 1), with little effect on growth of L-02 cells, and when IC_{50} were measured as 8.45 μmol/L and 191.55 μmol/L, respectively, the potency of ADFMChR to HepG2 cells was found to be similar to 5-fluorouracil (5-FU, IC_{50} was 9.27 μmol/L). The selective index of ADFMChR cytotoxicity to HepG2 cells was 22.67 (191.55/8.45), higher than 5-FU (SI was 7.05 (65.37/9.27).

Analysis of the effect of ADFMChR on apoptosis of HepG2 cell lines by FCM with PI staining

FCM with PI staining demonstrated that the apoptosis rates of HepG2 cells treated with 3.0, 10.0 and 30.0 μmol/L ADFMChR for 48 h were 5.79%, 9.29% and 37.8%, respectively, and were significantly higher when treated with 30.0 μmol/L ADFMChR than when treated with 30.0 μmol/L ChR (16.0%) ($P < 0.05$) and were similar to those obtained with 30.0 μmol/L 5-FU (41.0%) (Figure 2).

Detection of ADFMChR-induced apoptosis of HepG2 cells by agarose gel electrophoresis

DNA agarose gel electrophoresis showed that treatment of HepG2 cells with 10.0 μmol/L ADFMChR for 48 h and 72 h resulted in typical DNA ladders, which could be eliminated or attenuated by treating with 10.0 μmol/L ADFMChR plus 10.0 μmol/L GW9662 for 48 h and 72 h (Figure 3).

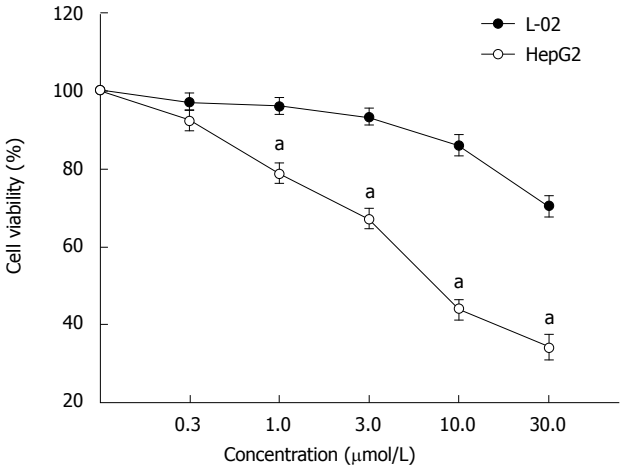

Figure 1 ADFMChR selectively inhibited proliferation of HepG2 cells. $^aP < 0.05$ *vs* treatment with ADFMChR in the same concentration to L-02 cells (mean ± SD, $n = 9$).

Analysis of the effect of ADFMChR on PPARγ, NF-κB, Bax and Bcl-2 protein expression of HepG2 cell line

Western blotting analysis showed that the relative densities of PPARγ, NF-κB, Bcl-2 and Bax protein bands of HepG2 cells treated with 3.0, 10.0, 30.0 μmol/L ADFMChR for 24 h were 109.3%, 126.4%, 147.7% and 92.9%, 89.0%, 72.4% and 94.1%, 85.5%, 77.3% and 106.8%, 116.3%, 125.7% of the HepG2 cells not treated with ADFMChR, respectively ($P < 0.05$) (Figure 4). This indicates that ADFMChR can increase the PPARγ and Bax protein expression and decrease NF-κB and Bcl-2 protein expression.

Effect of GW9662 on regulation of PPARγ and NF-κB protein expression by ADFMChR

Western blotting analysis demonstrated that when HepG2 cells were pre-incubated with 10.0 μmol/L GW9662, a blocker of PPARγ, for 30 min, the effects of 3.0, 30.0 μmol/L ADFMChR on PPARγ protein expression and NF-κB protein expression were antagonized or weakened (Figure 5), suggesting that the effects of ADFMChR on up-regulation of PPARγ protein expression and down-regulation of NF-κB protein expression were associated with the activation of PPARγ.

DISCUSSION

Tumorigenesis and tumor progression are strongly associated with abnormal apoptosis. A number of antitumor drugs exert their therapeutic effects by inducing or promoting apoptosis. Enhancing the antitumor effect of existing anticancer drugs, but not to increase its toxicity, is the aim of current anticancer research. There is evidence to support the concept that luteolin, apigenin and chrysin have great potential to be developed into novel cancer preventative agents[18].

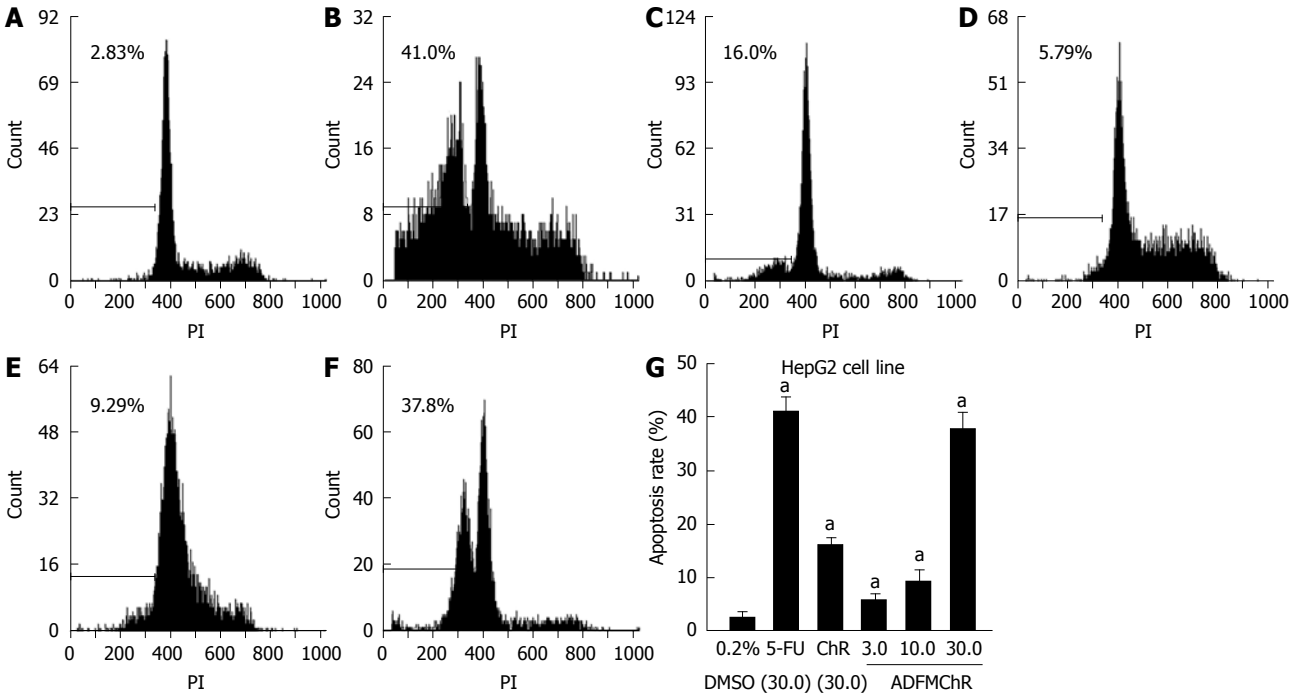

Figure 2 Induction of apoptosis of HepG2 cells by ADFMChR. A: Treated with 0.2% DMSO; B: Treated with 30.0 μmol/L 5-FU; C: Treated with 30.0 μmol/L ChR; D: Treated with 3.0 μmol/L ADFMChR; E: Treated with 10.0 μmol/L ADFMChR; F: Treated with 30.0 μmol/L ADFMChR; G: Quantification of induction of apoptosis analysis of HepG2 cells. $^aP < 0.05$ *vs* treatment with DMSO (mean ± SD, $n = 3$).

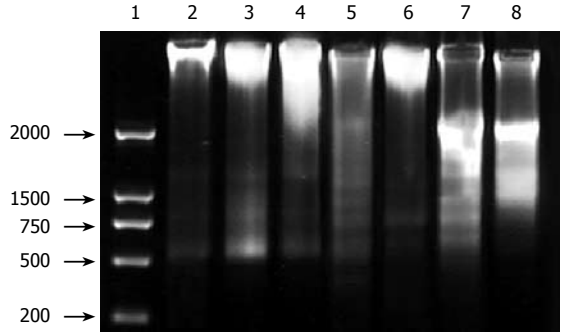

Figure 3 DNA ladder assay showing ADFMChR-induced apoptosis of HepG2 cells. Lane 1: DNA marker; lane 2: Control; lane 3: 10.0 μmol/L ADFMChR (24 h); lane 4: 10.0 μmol/L ADFMChR + GW9662 (24 h); lane 5: 10.0 μmol/L ADFMChR (48 h); lane 6: 10.0 μmol/L ADFMChR + GW9662 (48 h); lane 7: 10.0 μmol/L ADFMChR (72 h); lane 8: 10.0 μmol/L ADFMChR + GW9662 (72 h).

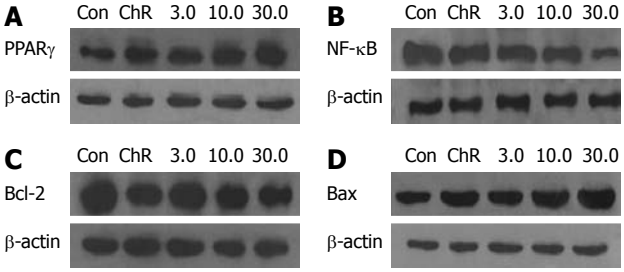

Figure 4 Western blotting analysis showing regulation of PPARγ (A), NF-κB (B), Bcl-2 (C) and Bax (D) protein expression in HepG2 cells by ADFMChR. (mean ± SD, $n = 3$).

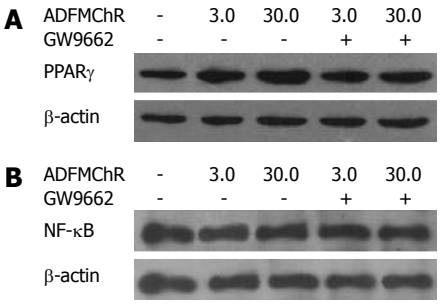

Figure 5 PPARγ antagonist GW9662 blocked the effects of ADFMChR on PPARγ and NF-κB protein expression in HepG2 cells. A: PPARγ; B: NF-κB. HepG2 cells were pretreated with 10.0 μmol/L GW9662 for 30 min, then exposed to 3.0, 30.0 μmol/L ADFMChR for 24 h, respectively (mean ± SD, $n = 3$).

Our previously research showed that ADFMChR potently inhibited the proliferation of ovarian cancer CoC1 cells in a dose-dependent manner[19], and could induce apoptosis of SMMC-7721 cells *in vitro*, with its mechanism possibly associated with G1 phase cell cycle arrest[20]. Li *et al*[19] and Xu *et al*[21] found that the ability of ADFMChR to induce induction of apoptosis in CoC1 cells may be mediated by activation of PPARγ, sequentially accompanied by reducing NF-κB and Bcl-2 levels and increasing Bax expression. Our experiment was to investigate the apoptosis of human liver carcinoma HepG2 cell line induced by ADFMChR and to provide experimental evidence for its application as an antitumor drug.

Apoptosis usually results in typical morphological and biochemical characteristics, including condensed chromatin in cells, appearance of apoptotic bodies, presence of hypodiploid peak in FCM analysis and DNA ladder bands on agarose electrophoresis[22,23]. In

this study, treatment of HepG2 cells with ADFMChR resulted in formation of DNA ladder bands and the appearance of marked hypodiploid peak. Thus, this experiment suggested that ADFMChR can induce apoptosis of human liver carcinoma HepG2 cell line *in vitro*.

PPARγ is a kind of ligand-activated nuclear transcription factor belonging to a nuclear receptor superfamily and has been implicated in metabolic diseases and is associated with cell proliferation, differentiation and apoptosis[24]. NF-κB inhibits apoptosis, promotes cell survival and reduces the expression of Bcl-2[25]. Chen *et al*[26] confirmed that PPARγ ligands may markedly inhibit NF-κB expression and reduce Bcl-2 expression leading to inhibited cell growth and induction of apoptosis of colonic cancer HT-29 cell line by activation of PPARγ. Liang *et al*[6] have recently shown that ChR is activated in different ways with thiazolidinones, and PPARγ inhibits activation of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase. 8-bromo-7-methoxychrysin (BrMChR) or 5,7-dihydroxy-8-nitrochrysin (NOChR) induce apoptosis of SGC2-7901 cell line by activating PPARγ[27,28]. In order to find out whether ADFMChR decreases NF-κB and Bcl-2 protein expression to induce apoptosis of HepG2 cells by activation of PPARγ, we pre-incubated HepG2 cells with GW9662, a selective antagonist of PPARγ, and observed the effect of ADFMChR on apoptosis and PPARγ and NF-κB protein expression of HepG2 cells. Our results showed that preincubation with GW9662 could effectively antagonize ADFMChR-induced apoptosis of HepG2 cells and down-regulation of NF-κB protein expression, suggesting that apoptosis of HepG2 cells induced by ADFMChR is dependent on activation of PPARγ.

Apoptosis is a complex process involving several genes, such as Bcl-2, Bax, and great attention has been given to the Bcl-2 family. The Bcl-2 family can positively and negatively regulate apoptosis[29]. Bcl-2 and Bax are two members of the Bcl-2 family, and play different roles in programmed cell death[30]. When Bax is over-expressed in cells, apoptosis in response to death signals is accelerated, leading to its designation as a death agonist[31]. When Bcl-2 is over-expressed it heterodimerizes with Bax and death is repressed[31]. Therefore, the ratio of Bcl-2 to Bax is important in determining susceptibility to apoptosis[30]. The results in this study confirmed that Bcl-2 expression in non-treated HepG2 cells was higher than in those treated with 3.0, 10.0, 30.0 μmol/L ADFMChR for 24 h; in contrast, Bax expression was lower. Thus, the ratio of Bcl-2 to Bax in HepG2 cells treated with ADFMChR was lower than that of non-treated HepG2 cells, which indicated that ADFMChR-induced HepG2 cells apoptosis was associated with down-regulation of Bcl-2 expression, up-regulation of Bax expression and reduction of the ratio of Bcl-2 to Bax.

In summary, ADFMChR possesses stronger anti-hepatic cancer effect *in vitro* than parent compound ChR, and was similar to 5-FU, and it exerts its apoptotic effect by activation of PPARγ, down-regulation of NF-κB and Bcl-2 protein expression, up-regulation of Bax protein expression, and reduction of the ratio of Bcl-2 to Bax. ADFMChR might be a promising candidate for the development of antitumor drugs.

COMMENTS

Background

Human liver carcinoma is the fifth most common cancer in the world. Unfortunately, the disease is often diagnosed at a late stage. For these patients, medical treatments, including chemotherapy, chemoembolization, ablation, and proton beam therapy, are not adequate. Most patients show disease recurrence that rapidly progresses to the advanced stages with multiple intrahepatic metastases and their 5-year relative survival rate is only 7%. Clearly, there is an urgent need for new therapies for this disease.

Research frontiers

Enhancing the antitumor effect of existing anticancer drugs, but not to increase its toxicity, is the aim of current anticancer research. Natural compounds have been extensively studied and have shown anti-carcinogenic activities by interfering with the initiation, development and progression of cancer through the modulation of various mechanisms including cellular proliferation, differentiation, apoptosis, angiogenesis, and metastasis. Flavonoids are a group of polyphenolic substances widely distributed in the plant kingdom and present in human diets. Previous reports have shown that flavonoids (such as chrysin, apigenin) could inhibit the proliferation and induce apoptosis in tumor cells. In this study, the authors demonstrate that 5-allyl-7-gen-difluoromethylenechrysin (ADFMChR) could induce apoptosis of human liver carcinoma HepG2 cells *in vitro* by activation of PPARγ.

Innovations and breakthroughs

Recent research has shown that chrysin and its derivatives possess a strong anticancer effect. This is the first study to report that ADFMChR, the derivative of chrysin, has a greater suppressive effect on proliferation of HepG2 cells than that of chrysin, and induces apoptosis of HepG2 cells. These data support the idea that ADFMChR has great potential to be developed into novel cancer preventative agents.

Applications

This finding may provide a molecular basis for the clinically observed cancer-preventive effects of 5-allyl-7-gen-difluoromethylenechrysin (ADFMChR) and new clues for research about pharmaceutical prevention and cure of human liver carcinoma.

Terminology

ADFMChR, a Chrysin derivative, which was taken as the principle compound to design and synthesize, was prepared by alkylation, methylation, and gen-difluoromethylation of chrysin, and was found to have stronger anticancer activities than parent compound chrysin.

Peer review

The authors demonstrate that the effects of ADFMChR on induction of apoptosis in HepG2 cells may be associated with activation of PPARγ, sequentially accompanied by inhibition of protein expression of NF-κB and Bcl-2 and reduced ratio of Bcl-2 to Bax. The results provide a new idea for cure of human liver carcinoma.

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BRIEF ARTICLES

No association between cyclooxygenase-2 and uridine diphosphate glucuronosyltransferase 1A6 genetic polymorphisms and colon cancer risk

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Author contributions: Thompson CL performed the statistical analyses, assisted with the subject recruitment and data collection and drafted the manuscript; Plummer SJ conducted some of the genotyping and assisted with the manuscript preparation; Merkulova A performed some of the genotyping; Tucker TC assisted with patient referrals and recruitment; Casey G coordinated the lab work and assisted with manuscript preparation; Li L led the study design and data collection and assisted with the manuscript preparation; Cheng I selected the SNPs for inclusion in the study and reviewed the manuscript.
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Abstract

AIM: To investigate the association of variations in the cyclooxygenase-2 (COX2) and uridine diphosphate glucuronosyltransferase 1A6 (UGT1A6) genes and non-steroidal anti-inflammatory drugs (NSAIDs) use with risk of colon cancer.

METHODS: NSAIDs, which are known to reduce the risk of colon cancer, act directly on COX2 and reduce its activity. Epidemiological studies have associated variations in the *COX2* gene with colon cancer risk, but others were unable to replicate this finding. Similarly, enzymes in the *UGT1A6* gene have been demonstrated to modify the therapeutic effect of NSAIDs on colon adenomas. Polymorphisms in the *UGT1A6* gene have been statistically shown to interact with NSAID intake to influence risk of developing colon adenomas, but not colon cancer. Here we examined the association of tagging single nucleotide polymorphisms (SNPs) in the *COX2* and *UGT1A6* genes, and their interaction with NSAID consumption, on risk of colon cancer in a population of 422 colon cancer cases and 481 population controls.

RESULTS: No SNP in either gene was individually statistically significantly associated with colon cancer, nor did they statistically significantly change the protective effect of NSAID consumption in our sample. Like others, we were unable to replicate the association of variants in the *COX2* gene with colon cancer risk ($P > 0.05$), and we did not observe that these variants modify the protective effect of NSAIDs ($P > 0.05$). We were able to confirm the lack of association of variants in *UGT1A6* with colon cancer risk, although further studies will have to be conducted to confirm the association of these variants with colon adenomas.

CONCLUSION: Our study does not support a role of COX2 and UGT1A6 genetic variations in the development of colon cancer.

Key words: Uridine diphosphate glucuronosyltransferase 1A6; Cyclooxygenase-2; Non-steroidal anti-inflammatory drugs; Colon cancer; Genetic association studies; Single nucleotide polymorphisms

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INTRODUCTION

Almost 150 000 new colorectal cancer cases are estimated to be diagnosed in 2008, resulting in almost 50 000 deaths (National Cancer Institute-www.cancer.gov). Colon adenomas (polyps) are a well established precursor of colon cancer. The genetic and environmental factors that cause the development of colon adenomas or their subsequent progression into cancer are not entirely known. Genetics are known to be a large risk factor for colon cancer, and indeed having a family history of colon cancer increases your risk of developing it yourself substantially. However, the known genetic susceptibility loci for colon cancer make up only a small fraction of this risk.

Cyclooxygenase-2 (COX-2) is a pro-inflammatory enzyme that converts arachidonic acid to prostaglandins. COX2 has been shown to be upregulated in a high proportion (about 86%) of human colorectal cancer[1]. Numerous additional findings have indicated a likely role for the cycloxygenases and inflammation in the development of colon cancer[2].

Non-steroidal anti-inflammatory drugs (NSAIDs), including aspirin and ibuprofen, act directly on COX2 as well as other targets to reduce activity. A substantial body of epidemiologic and randomized clinical trial evidence suggests that regular NSAID use and selective COX-2 inhibitors reduce the risks of colorectal cancer or the recurrence of adenomatous polyps[3], which is, at least in part, attributed to their known anti-inflammatory effects. The *COX-2* gene is thus a good candidate gene for colorectal carcinogenesis and its genetic variants may affect the susceptibility to the development of this colorectal cancer by altering the effects of this enzyme on the inflammatory response. To date, a few studies have evaluated a limited number of polymorphisms in the *COX2* gene in relation to risk of colorectal cancer and adenomatous polyps[4-7]. The findings have yielded inconsistent results, with some[4-6], but not all[7], reporting an association with the risk of colorectal cancer or polyps. Additionally, a sib-pair study of colon neoplasia with relatively small sample size found no linkage to the *COX2* locus[8].

Furthermore, genetic variation in the uridine diphosphate glucuronosyltransferase 1A6 (*UGT1A6*) gene have been associated with differences in aspirin metabolism, and those with the less frequent variants have a 30%-50% lower enzyme activity[9]. Additionally, a few studies have examined the association of genetic polymorphisms in *UGT1A6*, a rate-limiting enzyme directly involved in aspirin metabolism, and their interactions with aspirin or NSAIDs in relation to colorectal cancer and polyps[10-13]. Although two studies of colon cancer observed no association with genetic variants in *UGT1A6*[12,13], two studies of colon adenomas identified variants in *UGT1A6* that modified the protective effect of aspirin[10,11]. Others have identified variants that influence the risk of adenoma recurrence[14]. To further investigate the role of *COX2* and *UGT1A6* in relation to the risk of colon cancer, we tested nine tag single nucleotide polymorphisms (SNPs) in *COX2* and two functional SNPs in *UGT1A6*[13] in a population-based case-control study of colon cancer.

MATERIALS AND METHODS

Study population

The details of the present study have been described elsewhere[15]. Briefly, colon cases (n = 422) were identified through the population-based Surveillance, Epidemiology and End Results (SEER) Kentucky Cancer Registry, and 481 population controls living in Kentucky were recruited *via* random digit dialing. Participants donated a blood sample for genetic analyses and completed a detailed self-administered lifestyle risk factor questionnaire that included information on NSAID use. Participation rates were 72.2% for cases and 62.5% for controls. All participants provided written informed consent. The study was approved by the Institutional Review Boards of the University of Kentucky, Lexington, and Case Western Reserve University/University Hospitals Case Medical Center.

Genotyping

We assessed the common genetic variation (SNPs with minor allele frequencies > 5%) of *COX-2* that spanned about 2 kb upstream of the transcription start site and about 1 kb downstream of the 3' untranslated region. Seven tag SNPs for COX-2 were selected to predict unmeasured SNPs ($r^2 > 0.8$) using publicly available genotype data for European populations from the International HapMap project (www.hapmap.org) and the Perlegen and Seattle SNP projects (http://gvs.gs.washington.edu/GVS). One common SNP, -899 G/C (rs20417), that was previously associated with colorectal cancer[9], and rs689470, which was previously associated with prostate cancer[16], were also included. Two functional non-synonymous SNPs [rs1105879 (R184S) and rs2070959 (T181A)] in UGT1A6[13] were selected. Genotyping was performed using the Taqman allelic discrimination assay with genotyping error < 0.1%, as described previously[15].

Statistical analyses

We evaluated the association between *COX-2* and *UGT1A6* genotypes and colon cancer risk using multivariate unconditional logistic regression models. Each SNP was evaluated assuming dominant, additive and recessive modes of inheritance. For all SNPs, the allele with the lower frequency was coded as the risk allele. For the dominant model, individuals with at least one copy of the risk allele were coded as 1, others were

coded as 0. For the recessive model, only those with two copies of the risk allele were coded as 1. In the additive model, individuals were coded with the number of risk alleles they possessed (0, 1 or 2). In our base models, we adjusted for age, gender, and race. For these analyses, age was defined as age at diagnosis for cases and age at recruitment for controls. We further controlled for family history of colorectal cancer, body mass index (BMI), regular NSAID use, alcohol consumption, smoking, and intensity of recreational physical activity in the 20 s, for those with available data. Regular NSAID use was defined as having reported intake of at aspirin or ibuprofen at least twice a week for a period of one month or longer.

To evaluate potential effect modification of NSAID use, we tested for multiplicative interaction by including the main effects and a cross-product term of SNP × NSAID use in the logistic regression models. All *P*-values were two-sided, and all analyses were undertaken with SAS software (version 9.1; SAS Institute, Inc., Cary, North Carolina).

RESULTS

The characteristics and genotypic distributions of this predominantly Caucasian study population are summarized in Table 1. All SNPs were found to be in Hardy-Weinberg equilibrium both in controls alone and in the entire sample. We found no statistically significant association between any of the nine *COX2* SNPs and two functional *UGT1A6* SNPs and colon cancer risk, regardless of the mode of inheritance (Table 2).

We further explored potential effect modification of the association by regular NSAID use, and found no evidence for interaction of any SNP (Table 2) in either gene ($P > 0.05$).

Since others[10] have reported that UGT1A6 variants modify the therapeutic effects of aspirin in a population of women only, we stratified our analyses by gender. We found very little differences in results with nothing significant in females or males only as well (results not shown).

DISCUSSION

In our analysis, we examined potential effect modification by regular NSAID use rather than aspirin alone, as other groups have done, due to the smaller number of aspirin alone users. To account for this, we repeated our analysis using aspirin use only (115 cases and 157 controls), and the results did not change materially (not shown), with no significant findings. However, the lack of association with aspirin alone may be due to the small sample size and thus lack of statistical power.

Our study had over 90% power to detect an OR ⩾ 1.75 at a two-sided $\alpha = 0.05$ for the polymorphisms studied here, and over 80% power to detect an OR ⩾ 1.60, assuming a dominant model of inheritance and allele frequency of 0.1. While we did comprehensively capture the common genetic variation across the *COX2* gene, we only evaluated two putative functional SNPs in *UGT1A6*, and were limited in our ability to make direct conclusions about the effect of other genetic variants in *UGT1A6*. It is possible that these variants have smaller effects on colorectal cancer susceptibility or the therapeutic effects of NSAID use that we were unable to detect with this study.

Table 1 Population characteristics *n* (%)

	Case (*n* = 422)	Control (*n* = 481)	*P*[1]
Age (mean ± SD) (yr)[2]	62.9 ± 10.6	57.9 ± 11.1	< 0.0001
Gender			0.0002
Female	203 (50.5)	304 (64.1)	
Male	199 (49.5)	178 (35.9)	
Race			
Caucasian	378 (93.6)	449 (93.2)	0.35
African-American	21 (4.4)	21 (5.2)	
Other	12 (2.5)	5 (1.2)	
BMI (mean ± SD) (kg/m^2)	29.2 ± 6.2	28.1 ± 6.1	< 0.0001
Family history[3]			0.0011
Yes	94 (24.0)	71 (15.2)	
No	297 (76.0)	395 (84.8)	
NSAID use			0.14
Regular	235 (64.2)	306 (69.1)	
Irregular/none	131 (35.8)	137 (30.9)	
Physical activity			0.008
None/low	111 (29.1)	113 (24.7)	
Moderate	106 (27.8)	98 (21.4)	
High	165 (43.2)	246 (53.8)	
Regular alcohol use			0.04
Ever	134 (34.8)	191 (41.7)	
Never	251 (65.2)	267 (58.3)	
Smoking			0.88
Ever	207 (53.6)	248 (54.2)	
Never	179 (46.4)	210 (45.9)	

[1]*P*-value of significance difference between cases and controls in χ^2 test (discrete variables and genotypes) or *t*-test (continuous); [2]Age at diagnosis for cases, and age at questionnaire completion for controls; [3]Family history of first-degree relatives with colorectal cancer.

It is important to note that NSAID use was based on self-report. Individuals may not accurately recall duration or frequency of NSAID intake. Nevertheless, our finding of a protective effect of NSAID use (OR = 0.69, 95% CI = 0.50-0.96, $P = 0.02$) is in agreement with its well-documented association with colon cancer[17], lending credibility to our questionnaire data.

In conclusion, this moderately large population-based case-control study did not detect a direct association between variants in the *UGT1A6* and *COX2* genes and risk of colon cancer nor an effect modification by NSAIDs. The results of our study are in line with the two studies of colon cancer showing null results[12,13]. Taken together with the studies showing an association with polyps[10,11], our results suggest genetic variation of *UGT1A6* may affect the early stages of colon tumorigenesis, but has little influence on the progression from adenomatous polyps to colon cancer, although we are unable to test that hypothesis directly.

Table 2 Odds ratios for individual SNPs and SNP by NSAID use interactions

SNP	Case/control	Crude		Adjusted		Stratified by NSAID use		
		OR (95% CI)	P^1	OR (95% CI)	P^2	Regular	None	P^3
COX2								
rs2066826			0.45		0.34			0.41
GG	314/370	Ref		Ref		Ref	Ref	
AG	98/99	1.17 (0.85, 1.60)		1.23 (0.88, 1.72)		1.05 (0.68, 2.64)	1.52 (0.82, 2.83)	
AA	8/12	0.79 (0.32, 1.95)		1.06 (0.41, 2.73)		0.52 (0.10, 2.64)	1.96 (0.30, 12.62)	
rs2206593			0.049		0.25			0.47
GG	382/417	Ref		Ref		Ref	Ref	
AG	39/63	0.68 (0.44, 1.03)		0.69 (0.44, 1.07)		0.64 (0.36, 1.13)	0.89 (0.42, 1.90)	
AA	0/2	----		----		----	----	
rs5277			0.97		0.58			0.76
CC	310/353	Ref		Ref		Ref	Ref	
CG	104/119	1.00 (0.73, 1.35)		0.90 (0.65, 1.25)		0.90 (0.59, 1.36)	0.86 (0.48, 1.54)	
GG	7/8	1.00 (0.36, 2.78)		0.80 (0.27, 2.36)		0.52 (0.09, 2.95)	1.85 (0.32, 10.56)	
rs689470			0.87		0.79			> 0.99
GG	380/440	Ref		Ref		Ref	Ref	
AG	35/36	1.13 (0.69, 1.83)		1.15 (0.66, 2.02)		1.13 (0.54, 2.35)	1.59 (0.60, 4.22)	
AA	4/4	1.16 (0.29, 4.66)		1.60 (0.26, 9.70)		----	2.38 (0.13, 43.43)	
rs4648310			0.95		0.67			0.50
AA	405/462	Ref		Ref		Ref	Ref	
AG	17/19	1.08 (0.55, 2.12)		1.07 (0.53, 2.19)		1.25 (0.48, 3.23)	0.69 (0.21, 2.27)	
GG	0/1	----		----		----	----	
rs5275			0.34		0.51			0.89
AA	176/216	Ref		Ref		Ref	Ref	
AG	189/199	1.17 (0.88, 1.55)		1.17 (0.87, 1.58)		1.14 (0.78, 1.67)	1.04 (0.60, 1.79)	
GG	56/65	1.06 (0.70, 1.59)		1.22 (0.79, 1.88)		0.98 (0.57, 1.71)	1.79 (0.78, 4.12)	
rs689466			0.46		0.69			0.99
AA	275/297	Ref		Ref		Ref	Ref	
AG	138/168	0.89 (0.67, 1.17)		0.86 (0.64, 1.15)		0.91 (0.62, 1.33)	0.83 (0.48, 1.42)	
GG	9/15	0.65 (0.28, 1.51)		0.65 (0.26, 1.60)		0.75 (0.26, 2.17)	0.69 (0.11, 4.45)	
rs20417			0.41		0.20			0.44
GG	291/343	Ref		Ref		Ref	Ref	
CG	119/121	1.16 (0.86, 1.56)		1.25 (0.91, 1.71)		1.17 (0.78, 1.74)	1.39 (0.78, 2.47)	
CC	11/15	0.86 (0.39, 1.91)		0.97 (0.43, 2.20)		0.46 (0.14, 1.54)	3.12 (0.57, 17.15)	
rs2745557			0.61		0.41			0.18
GG	287/321	Ref		Ref		Ref	Ref	
AG	120/141	0.95 (0.71, 1.27)		0.93 (0.69, 1.27)		1.04 (0.70, 1.54)	0.77 (0.43, 1.38)	
AA	13/19	0.76 (0.37, 1.58)		1.01 (0.48, 2.14)		1.49 (0.60, 3.67)	0.34 (0.07, 1.68)	
UGT1A6								
rs1105879			0.46		0.28			0.93
AA	191/206	Ref		Ref		Ref	Ref	
AC	167/209	0.86 (0.65, 1.14)		0.84 (0.63, 1.14)		0.84 (0.57, 1.23)	0.86 (0.50, 1.48)	
CC	64/66	1.05 (0.70, 1.55)		1.07 (0.71, 1.64)		1.20 (0.70, 2.06)	1.07 (0.50, 2.32)	
rs2070959			0.21		0.21			0.93
AA	208/207	Ref		Ref		Ref	Ref	
AG	154/206	0.78 (0.59, 1.03)		0.78 (0.58, 1.04)		0.76 (0.52, 1.12)	0.82 (0.48, 1.42)	
GG	59/57	1.08 (0.72, 1.63)		1.11 (0.72, 1.72)		1.26 (0.72, 2.23)	1.04 (0.47, 2.29)	

[1]*P*-value of SNP in best fitting genetic model in the unadjusted logistic regression; [2]*P*-value of SNP in best fitting genetic model in the full multivariate logistic regression; [3]*P*-value of interaction of SNP in best fitting genetic model with regular NSAID use in the logistic regression.

COMMENTS

Background

Colon cancer accounts for almost 150 000 cancer cases and 50 000 deaths in the United States alone. Non-steroidal anti-inflammatory drug (NSAID) use has been well known to reduce the risk of colon cancer.

Research frontiers

It is unclear how individual variation influences the protective effect of NSAIDs. Cyclooxygenase-2 (*COX2*) and uridine diphosphate glucuronosyltransferase 1A6 (*UGT1A6*) are two genes that have been proposed to modify the effect of NSAIDs on colon cancer risk. *COX2* is a direct target of NSAIDs and UGT1A6 variations have been shown to alter the metabolism of aspirin, a common NSAID.

Innovations and breakthroughs

This study has provided further insight into the role of the *COX2* and *UGT1A6* genes in colon cancer risk.

Applications

Since genetic variation often accounts for differences in an individual's response to preventive or therapeutic drugs, it is important to understand the relationship between genes, the drugs and the intended outcome. NSAIDs have been suggested as a chemopreventive agent for individuals at high risk of colon cancer. It is thus important to identify those individuals who would most benefit from the use of NSAIDs. This study found that, while other genes may predict enhanced benefit of NSAID use for colon cancer prevention, *COX2* and *UGT1A6* do not.

Terminology

COX2 and *UGT1A6* are two genes involved in NSAID metabolism. Since NSAIDs, including aspirin and ibuprofen, are known to be protective for colon cancer, it has been hypothesized that these genes would influence and individual's response to NSAID use with respect to colon cancer risk.

Peer review

The authors examined the association of *COX2* and *UGT1A6* polymorphisms

and risk of colon cancer. They also evaluated the effect of variations in these genes on the protective effect of NSAIDs. They did not find an association with any variant examined and risk of colon cancer nor did they find they these variants altered NSAID effects. This study gives further evidence that these genes are not directly involved in colon cancer carcinogenesis.

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Contrast-enhanced sonography *versus* biopsy for the differential diagnosis of thrombosis in hepatocellular carcinoma patients

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Abstract

AIM: To clarify which method has accuracy: 2nd generation contrast-enhanced ultrasound or biopsy of portal vein thrombus in the differential diagnosis of portal vein thrombosis.

METHODS: One hundred and eighty-six patients with hepatocellular carcinoma and portal vein thrombosis underwent in blinded fashion a 2nd generation contrast-enhanced ultrasound and biopsy of portal vein thrombus; both results were examined on the basis of the follow-up of patients compared to reference-standard.

RESULTS: One hundred and eight patients completed the study. Benign thrombosis on 2nd generation contrast-enhanced ultrasound was characterised by progressive hypoenhancing of the thrombus; in malignant portal vein thrombosis there was a precocious homogeneous enhancement of the thrombus. On follow-up there were 50 of 108 patients with benign thrombosis: all were correctly diagnosed by both methods. There were 58 of 108 patients with malignant thrombosis: amongst these, 52 were correctly diagnosed by both methods, the remainder did not present malignant cells on portal vein thrombus biopsy and showed on 2nd generation contrast-enhanced ultrasound an inhomogeneous enhancement pattern. A new biopsy during the follow-up, guided to the area of thrombus that showed up on 2nd generation contrast-enhanced ultrasound, demonstrated an enhancing pattern indicating malignant cells.

CONCLUSION: In patients with hepatocellular carcinoma complicated by portal vein thrombosis, 2nd generation contrast-enhanced ultrasound of portal vein thrombus is very useful in assessing the benign or malignant nature of the thrombus. Puncture biopsy of thrombus is usually accurate but presents some sampling errors, so, when pathological results are required, 2nd generation contrast-enhanced ultrasound could guide the sampling needle to the correct area of the thrombus.

Key words: Hepatocellular carcinoma; 2nd generation contrast enhanced ultrasound; Contrast enhanced sonography; Malignant thrombosis; Portal vein biopsy

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INTRODUCTION

About 20% of patients at first access visit to a specialized centre for the care of hepatocellular carcinoma (HCC)[1] are in need of differential diagnosis between be-

nign portal vein thrombosis (PVT) or malignant thrombosis. The nature of the thrombus can have a significant impact on treatment. In particular, because the prevalence of tumor recurrence is nearly 100%, patients who have HCC and proven neoplastic vascular thrombus are not candidates for any treatment[2-5]. Malignant PVT can occur in patients with cirrhosis, with or without the presence of parenchymal HCC, because there is the possibility of intravascular first growth of this neoplasm[6]. Thrombi have been studied in an effort to determine imaging characteristics that could be used to distinguish benign from malignant thrombi[7-11]. Unfortunately, the imaging characteristics tend to overlap, in particular on computer tomography (CT) or magnetic resonance imagine (MRI) exams only the feature of thrombus-tumor continuity is widely accepted as a reliable indicator of thrombus malignancy[12,13]. In patients where percutaneous ablation of HCC is the therapy of choice, the technique of reference throughout the world for differentiating benign from malignant PVT is percutaneous fine needle biopsy (FNB) of the thrombus[14]. Given the obvious clinical utility of a reliable non-invasive technique for diagnosis of malignant PVT, the limitation of previous imaging studies and an opportunity at our institutions to perform a reasonably large prospective study with cytopathologic correlation in all patients, we undertook an investigation to compare Contrast-Enhanced Sonography (CEUS) and portal vein FNB of thrombus in differentiating benign from malignant thrombosis.

MATERIALS AND METHODS

The study protocol which was fully concordant with ethical principles of the Declaration Helsinki was approved by the institutional ethic committee. A written informed consent was obtained from each patient.

Patients

From January 2001 to February 2006, we enrolled consecutively 256 cirrhotic patients with HCC and PVT (Table 1). The major part of these patients were not eligible for surgical resection/liver transplantation, the others refused intervention. We restricted analysis only to patients without direct contiguity between the thrombus and HCC and considering also the patients drop out on follow-up we completed the study in 108 patients. Clinical and ultrasonography (US) details of these patients are displayed in Table 2.

Study design

Diagnosis of HCC was made according to the guidelines drawn up the Barcelona 2000 EASL Conference[15]. These guidelines suggest that in a liver cirrhosis setting HCC may be diagnosed by coincidental findings in at least two imaging modalities (spiral CT and Doppler US or MRI) that should reveal arterial hypervascularity or in the case of combined criteria (spiral CT with alfa-fetoprotein levels > 400 ng/mL). US guided biopsy should be performed in those cases in which the above-mentioned criteria are not satisfied[15]. Pathological diagnosis of HCC was made according to the International Working Party criteria[16]. The thrombi were detected on routine sonographic and CT examination. Spiral CT was performed in a range of one month before or after color Doppler US. In patients after diagnosis of HCC, in order to characterize PVT, we performed both CEUS and portal FNB; according to the results of portal FNB patients were evaluated for potential percutaneous ablation of HCC. Study design is displayed in Figure 1. Patients underwent first CEUS then portal FNB on the same occasion carried out by two separate operators; the operator that performed PVT FNB was blinded to the results of CEUS. Patients without malignant cells on FNB underwent percutaneous treatment; the others underwent supportive care. Results of baseline CEUS were evaluated in blind fashion on the basis of the evolution

Table 1 Enrollment design

Contents	
Inclusion criteria	Presence of one to three focal HCC Presence of intra-vascular[1] portal vein thrombosis Child-Pugh class A or B
Patients initially enrolled	Men: 190 Women: 66
Excluded for US evidence direct HCC portal vein invasion	70
Drop out on follow-up	78 (42%)
Died	30
Liver could not be adequately visualized	9
Patients studied	Mean age: 66 ± 6 Men: 82 Women: 26

[1]Not US features of infiltration of perivascular parenchyma: intact vessel wall.

Table 2 Principal clinical/ultrasound features of patients

Clinical data	Results
Child A/B	44/64
Etiology	
HCV related	58
HBV related	23
Alcohol related	12
Mixed etiology	15
Number of HCC nodules	
Single nodule	10
Median size	44 mm (range 40-75 mm)
Two nodules	22
Median size	41 mm (range 33-68 mm)
Three nodules	76
Median size	39 mm (range 32-67 mm)
Topography of portal vein thrombosis	
In right or left but not in the main portal vein	80 (74%)
In main, right and left portal vein	14 (13%)
In right or left and main portal vein	12 (11%)
In main portal vein	2 (1.8%)
Complete or incomplete vessel occlusion of on power-color-Doppler	
A complete occlusion of the portal vessel	99 (91%)
Incomplete thrombosis lumen	9 (9%)

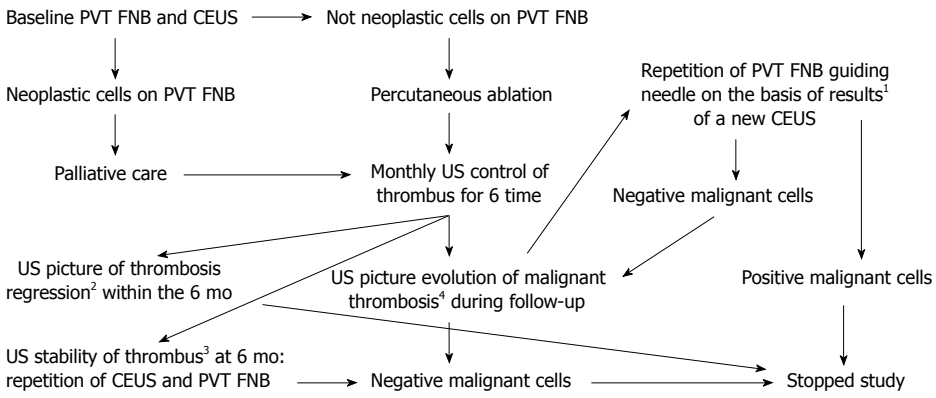

Figure 1 Study design. PVT FNB: Portal vein thrombus fine needle biopsy; CEUS: 2nd generation Contrast-Enhanced US (CEUS) of thrombus; [1]Guiding needle on portion of thrombus showing on CEUS precocious iso or hyperenhancement pattern; [2]No increase in size and distribution with preservation of vessel wall or recanalization/shrinkage, or disappearance of a PVT within the 6 mo of follow-up were accepted as proof of a benign portal vein thrombus; [3]No change in feature of thrombus and in the diameter of the segment of involved vein at 6 mo of follow-up; [4]Increase in size with infiltration of perivascular parenchyma and interruption of vessel wall was US features indicative of malignant thrombosis.

of thrombus on follow-up and were not decisive for the therapeutic management of patients. All patients after CEUS and PVT FNB were followed up for 6 mo; they underwent monthly US examination by an operator that was blinded to CEUS and PVT FNB initial results. We considered as the reference standard of benign or malignant thrombosis the US evolution of thrombus in combination with concordant cytology on new PVT FNB: i.e. no increase in size and distribution with preservation of vessel wall or recanalization/shrinkage, or disappearance of a PVT within the sixth months of follow-up were accepted as proof of a benign portal vein thrombus. However, in cases of stability of thrombi with no change in diameter of the segment of involved vein at 6 mo of follow-up, patients were resubmitted to CEUS and PVT FNB; in absence of malignant cells at this cytological examination, thrombus was definitively considered benign. Our reference standard of malignant thrombosis was increase in size on US, with or without infiltration of perivascular parenchyma and interruption of vessel wall at any time point during the follow-up. In the presence of such evolution of the US picture, CEUS and PVT FNB were repeated with guidance of the needle biopsy to thrombus areas with enhancing pattern allowing for the search for malignant cells; in presence of these, patients stopped the follow-up and thrombus was definitively considered malignant. Patients that died on follow-up without definitive diagnosis were considered drop outs. Specimens were obtained with a 22-Gauge Chiba needle in all patients; needles are manufactured with a removable occlusive stylet. The same biopsy technique described by others[14] was used in all patients. A positive result was considered if the biopsy specimen contained hepatocytes that had malignant features.

Baseline and contrast-enhanced harmonic ultrasound

An Aloka-Prosounds-5500-model equipped with a multifrequencies 2-6 MHz sector probe, was used. Contrast-enhanced imaging was performed according to the protocol used for the Bracco-SonoVue preclinical trial[17]. Examination was performed with low acoustic power (mechanical index under 0.01). SonoVue (BR1; Bracco, Milan, Italy)[18,19] consisted of sulfur-hexafluoride (SF_6) vapor-filled and phospholipid-stabilized microbubbles with a diameter uniformly smaller than 8 μm; these microbubbles circulate in the intravascular space crossing pulmonary and systemic capillary circulation[20,21]. 2.5 mL of contrast-agent were administered for each patient. Thanks to its ability to avoid destruction of bubbles, the low mechanical index technique allows identification of the entire vascular phase of contrast agent perfusion, consisting of the arterial phase (15-30 s after injection of agent), the portal phase (30-60 s after injection of agent) and the late parenchymal phase[22-24]. Positive arterial enhancement of the thrombus was defined as a greater hyperechogenicity of the vascular bed-occupying lesion in comparison to the surrounding liver parenchyma detected during the arterial phase. Two independent highly experienced readers firstly performed off-site assessments of the videotapes in a computer-generated randomised order. The readers were blinded to all clinical and pathological information as to the nature of the analysed thrombi.

Statistical analysis

Sensitivity, specificity, positive and negative predictive values of CEUS and PVT FNB were obtained for diagnosis of the nature of the thrombus; we considered as reference standard the US picture evolution of thrombus on follow-up, with a new PVT FNB as above decrypted accordingly to obtain definitive cytological confirmation.

RESULTS

On follow-up we identified 58 of 108 patients (53.7%) with malignant thrombosis and 50 (46.3%) with benign thrombosis. Figure 2 displayed results of combined tests: in 50 of 56 patients without malignant cells on first PVT FNB, benign PVT was characterized on CEUS by a diffuse homogeneous hypoenhancing pattern and this appearance was persistent compared with the adjacent liver, also during late phase (Figure 3A-C). In the follow up of

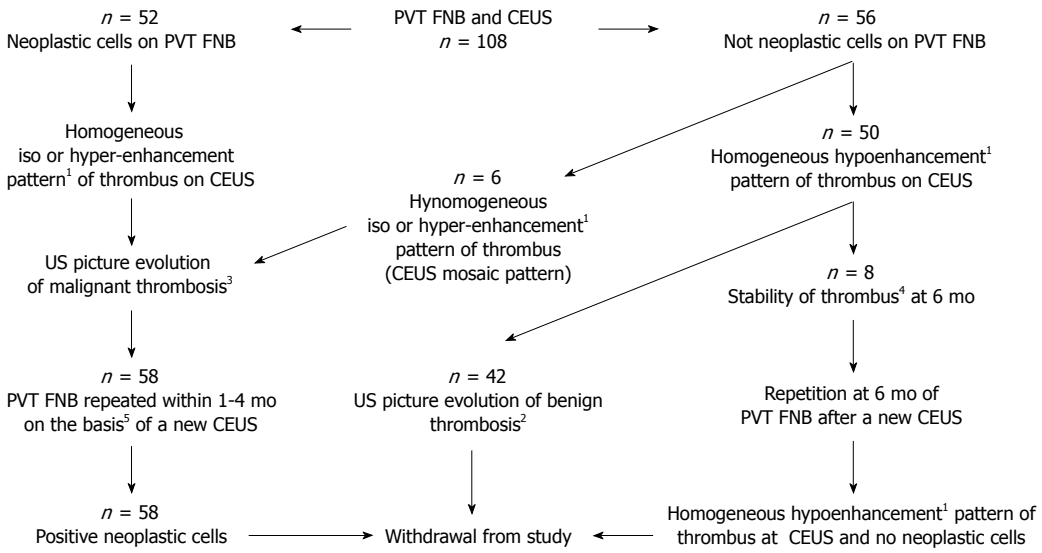

Figure 2 Summary of combined test results. [1]Iso, hyper, or hypoenhancement pattern of thrombus compared to the surrounding parenchyma; [2]Reference standard of benign thrombosis is a US evidence of evolving thrombus: no increase in size or distribution with vessel wall preservation or recanalization/shrinkage, or disappearance of a PVT within the 6 mo of follow-up were accepted as evidence of a benign portal vein thrombus; [3]Us image of evolution, indicating malignant thrombosis: increase in size with infiltration of perivascular parenchyma and interruption of vessel wall was US features of malignant thrombosis; [4]No change in thrombus image and in the diameter of the segment of vein involved at 6 mo of follow-up; [5]PVT FNB were repeated guiding the needle to the thrombus territories with enhancing pattern.

these patients we observed 16 spontaneous disappearances of thrombi after treatment of HCC, 26 recanalization with shrinkage of thrombi and 8 cases of stability of thrombi with no change in diameter of the segment of involved vein. These benign PVT patients were resubmitted to CEUS and PVT FNB at 6 mo with the same combined results as at the start (Figure 2). In 6 patients of 56 without malignant cells on FNB (false negative on PVT FNB), CEUS showed no homogeneous arterial enhancement of some small portions of thrombus. On follow-up the thrombus of these patients showed intravascular spread with growth in maximal diameter of the involved segments of the portal branch from a mean of 8 mm to a mean of 14 mm, with interruption of the vessel wall in 3 patients; we repeated CEUS and guided a new portal FNB to areas of thrombus that showed an enhancing pattern, obtaining positive results for malignant hepatocytes. The islands of neoplastic tissue were located at baseline CEUS as corresponding to the anterior wall of right branch in 1 case, corresponding to and mainly in the centre of the vessel in 3 cases, and corresponding to the posterior wall of left portal branch in 2 cases; they measured between 9 mm and 15 mm in length. In all 6 cases there was a complete thrombosis, involving the portal trunk and both branches, which measured in length between 18 mm and 27 mm. We retrospectively called the CEUS appearance of these cases "mosaic picture" of neoplastic thrombus (Figure 4A and B).

There were 52 patients (Figure 2) with the presence of malignant cells on the baseline portal FNB: these showed on follow-up growth in diameter and intravascular spread of PVT within 1-4 mo. The repetition of PVB FNB in all these patients, with an US picture evolution of malignant thrombosis on the basis of a new CEUS, always confirmed the diagnosis (not false-negative). Typical malignant PVT had an unequivocal appearance at CEUS: during the arterial phase intense and diffuse homogeneous contrast enhancement (Figure 5A and B) was seen, followed or not by a washout of contrast material from the thrombus; the appearance was iso or hyperechoic in arterial phase and hypo or isoechoic during the late phase (Table 3). Sensitivity, specificity, positive and negative predictive value of PVT FNB and CEUS were the same for both, respectively: 89.6%, 100%, 100%, 89.2%. These

Table 3 Contingent tables

Group	Results
Patients studied on follow-up	108
Malignant thrombosis	58 (53.7%)
Benign thrombosis	50 (46.3%)
Presence of neoplastic cells on PVT FNB[1]	
True positive	52 (48.1%)
False positive	0
Not neoplastic cells on baseline PVT FNB	
False negative	6[2] (5%)
True negative	50 (46.3%)
Iso-hyper-enhancement pattern[1] on CEUS and mosaic pattern	
True positive	58
Precocious isoenhancement pattern	21
Precocious hyperenhancement pattern	31
Mosaic pattern[3]	6
False positive	0
Hypoenhancement patternc on CEUS	
False negative	0
True negative	50 (46.3%)

Sensitivity, specificity, positive and negative predictive value of CEUS: All 100%. [1]Iso-hyper-hypoenhancement pattern of thrombus respect to surround parenchyma; [2]False negative patients on portal vein FNB were the same with mosaic pattern on CEUS; [3]Hynomogeneous iso-hyperenhancement of thrombus.

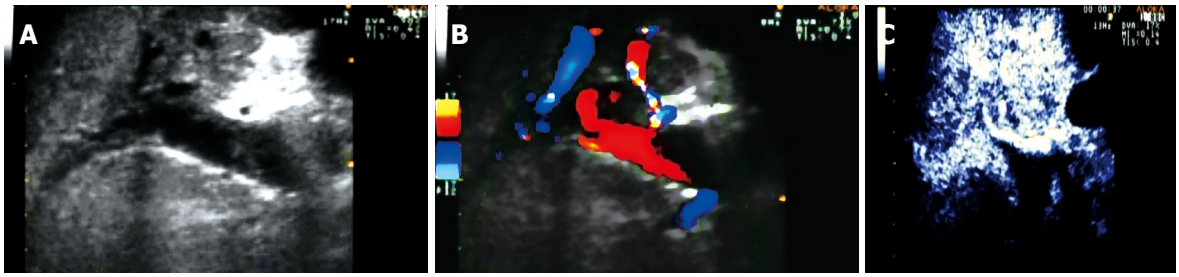

Figure 3 Benign thrombus. A: On sonography lumen of portal vein is partially filled with hypoechoic material representing occlusive thrombus; B: Color Doppler ultrasound reveals color signals only within a portion of portal lumen; C: Contrast-enhanced sonography scan during portal phase reveals uniformly non-enhancing area within portal vein, perfectly reproducing the benign thrombus.

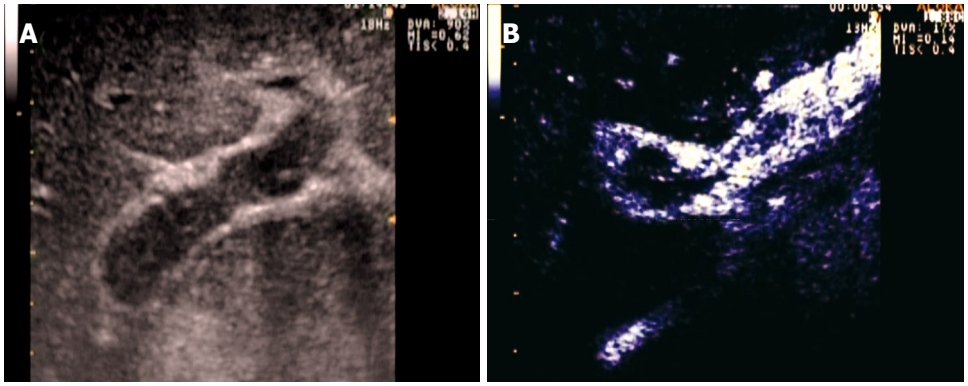

Figure 4 Malignant mosaic thrombus. A: Sonography scan reveals isoechoic area within portal lumen representing thrombus; B: Contrast enhanced sonography scan during late arterial phase reveals thrombus as predominantly enhancing area, indicative of arterial neovascularization (malignant thrombosis) with some non-enhancing areas of the thrombus (mosaic pattern).

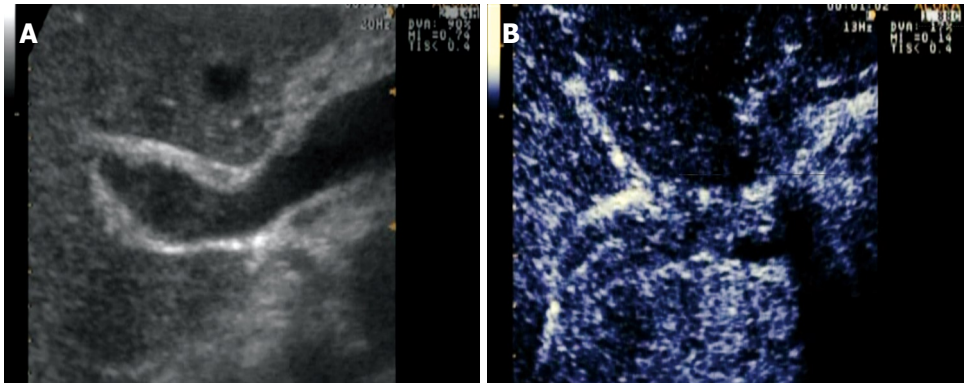

Figure 5 Malignant thrombus. A: sonography reveals echogenic area (thrombus) within vessel lumen; B: during arterial phase of contrast-enhanced sonography the diffusely enhanced area representing thrombus with internal neovascularity.

values coincided for both techniques because, as shown in Table 3, the false-negative patients on baseline CEUS and PVT FNB were the same. On the other hand, if we retrospectively admitted the mosaic picture of enhancement (the picture of the 6 false-negative patients on CEUS) as an alternative, but possible, picture of appearance of malignant PVT on CEUS, and considering that prospectively no false-positive or false-negative results were given, 100% of sensitivity and specificity were obtained for this technique.

DISCUSSION

In previous studies, we[25] and others[26] have described the usefulness and superiority of contrast-enhanced sonography with respect to sonography and color Doppler sonography in the detection and characterisation of thrombus. Here our study differs in two points: (1) we systematically compared in blinded fashion the validity of portal FNB with respect to contrast enhancement of portal thrombus; (2) we excluded from our study patients with evidence of continuity between thrombus and tumor tissue (most of patients in study of Rossi *et al*[26]) a feature considered diagnostic of malignant thrombosis both on sonography[26], and on helical TC/MRI imaging[12,13].

CT remains the primary imaging technique for staging HCC and identifying PVT[27]. MRI also appears to be a promising tool[28]. Although the capacity for CT to show main or lobar PVT is well established, controversy surrounds radiologists' ability to use CT to consistently differentiate between malignant and simple thrombi[3,7,27]. This reluctance to stage possible portal vein invasion by CT/MRI alone has perhaps been appropriate given the lack of a formal study in the literature that compares the imaging characteristics of proven benign and malignant thrombi. It was shown that tumor thrombus neovascularity may also be identified, with variable accuracy, by color Doppler

sonography[29-34]. Now the use of CEUS permits us to study in real time micro-vascular architecture of each thrombus, searching for global arterial enhancement typical of HCC neovascularity. The interpretation of results is based on general characteristics of enhancing/hypoenhancing of thrombus after administration of contrast ultrasound agent. The sensitivity of CEUS is better with respect to Doppler sampling of intrathrombus vessels; there are in fact technical limits of Doppler sampling due to the small diameter of vessels of the microvascular architecture of neoplastic tissue[35].

We deduced that homogeneous hypoenhancement of the thrombus on CEUS with respect to the surrounding parenchyma is diagnostic for benign thrombosis. Significantly, benign PVT does not show enhancement at any time after ultrasound contrast agent administration. The homogeneous enhancement of thrombus on CEUS must be considered diagnostic for malignant thrombosis. In particular, the appearance of malignant PVT can be precociously hyperechoic or isoechoic with respect to the surrounding parenchyma: this picture could be due to diffuse arterialization of surrounding liver parenchyma, a pathophysiological phenomenon secondary to the same thrombosis.

In our study, in order to obtain an accurate differential diagnosis as to the nature of a PVT, we utilized as gold-standard methods the prospective evaluation of the thrombus with, in most cases, a concordant cytology on PVT FNB repetition. We in fact were uncertain about the validity of using only baseline portal FNB to determine diagnosis because of the not optimal sensitivity of the method. The possibility of sampling error could result in both false-positive and false-negative diagnoses for malignant PVT. Because a benign thrombus does not contain hepatocytes, specimens that include cells from the periportal hepatic parenchyma or hepatocytes picked up during passage of the biopsy needle through the liver could lead to false-positive diagnoses of malignant tumor. We prevented false-positive diagnoses by using a biopsy needle with an occlusive stylet, keeping the stylet tightly seated until the needle tip was detected inside the portal vein and performing the biopsy under continuous sonographic visualization with the needle tip kept within the lumen of the portal vein at all times during passages. In our study no diagnosis of malignant tumor on FNB PVT was false-positive indicating that the invasive procedure is maximally specific. False-negative diagnoses for malignant cells could be produced if the portion of a malignant portal vein thrombus from which a specimen was obtained failed to contain malignant hepatocytes. We tried to prevent false-negative diagnoses by performing the biopsy on the portal vein thrombus by sampling the longest possible segment of a portal vein thrombus. We obtained anyway 6 false-negative results for malignant thrombi. In all 6 cases the appearance on CEUS was as an inhomogeneous enhancement of the thrombus; we called the CEUS appearance of these cases "mosaic-picture" of neoplastic thrombus. We were unhappy about the false-negative results of portal FNB derived from sampling the non-neoplastic portion of the thrombus; we repeated portal FNB within 1-4 mo guiding the biopsy on results of the CEUS (FNB of enhancing part of thrombus) and obtained malignant cells. We supposed that the echotexture of malignant thrombus on CEUS was not homogeneous in these 6 patients because there were some occult islands of neoplastic tissue in the thrombus that after administration of ultrasound contrast agent showed as enhancing patterns with respect to the diffuse hypoenhancing of the remaining benign thrombus. Probably in these cases the phenomena of benign thrombosis was superimposed on the initial neoplastic invasion of the portal vein. So CEUS of portal vein thrombi appears as a diagnostic procedure more accurate than "blind" portal FNB in the diagnosis of malignant thrombosis with regard to the possibility of giving a panoramic vision of the thrombus without the sampling-error of "blind" portal FNB. So it is reasonable, when cytology confirmation of malignant thrombosis is needed, that portal FNB can be guided on the result of CEUS in order to reduce false-negative results due to casual sampling.

In conclusion, CEUS of portal thrombus is more accurate than biopsy of thrombus for making the differential diagnosis as to the nature of the thrombus. CEUS of portal thrombus is a reliable diagnostic tool for assessing non-invasively the nature of the PVT. This procedure is usually accurate but presents some sampling errors linked to the 'blind' biopsy of the thrombus.

COMMENTS

Background

About 20% of patients at first access visit to a specialized centre on the care of hepatocellular carcinoma need differential diagnosis between benign portal vein thrombosis (PVT) or malignant thrombosis. Patients who have hepatocellular carcinoma and proven neoplastic vascular thrombus are not candidates for any treatment: in these cases the prevalence of tumor recurrence is nearly 100%.

Research frontiers

The world technique of reference for differentiating benign from malignant PVT is the invasive percutaneous fine needle biopsy (FNB) of the thrombus. Given the obvious clinical utility of a reliable non-invasive technique for the diagnosis of malignant PVT, the authors undertook an investigation to compare Contrast-Enhanced Sonography (CEUS) and portal vein FNB of thrombus for differentiating benign from malignant thrombosis.

Innovations and breakthroughs

For the first time, the authors systematically compare in blinded fashion the validity of portal FNB with respect to non-invasive contrast enhancement of portal thrombus in order to differentiate benign from malignant thrombosis.

Applications

CEUS of portal thrombus is more accurate than biopsy of thrombus for making differential diagnosis of the nature of the thrombus. CEUS of portal thrombus is a reliable diagnostic tool for assessing non-invasively the nature of PVT.

Terminology

CEUS consists of an ultrasound exam which is performed after parenteral administration of an ultrasound contrast. The CEUS in this study consists of sulfurhexafluoride (SF_6) vapor-filled and phospholipid-stabilized microbubbles with a diameter uniformly smaller than 8 μm; these microbubbles circulate in the intravascular space crossing pulmonary and systemic capillary circulation.

Peer review

This paper addresses the value of II Generation CEUS in non-invasive differential diagnosis of benign from malign portal vein thrombosis. The manuscript is interesting.

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BRIEF ARTICLES

Estimating glomerular filtration rate preoperatively for patients undergoing hepatectomy

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Abstract

AIM: To compare creatinine clearance (Ccr) with estimated glomerular filtration rate (eGFR) in preoperative renal function tests in patients undergoing hepatectomy.

METHODS: The records of 197 patients undergoing hepatectomy between August 2006 and August 2008 were studied, and preoperative Ccr, a three-variable equation for eGFR (eGFR3) and a five-variable equation for eGFR (eGFR5) were calculated. Abnormal values were defined as Ccr < 50 mL/min, eGFR3 and eGFR5 < 60 mL/min per 1.73 m^2. The maximum increases in the postoperative serum creatinine (post Cr) level and postoperative rate of increase in the serum Cr level (post Cr rate) were compared.

RESULTS: There were 37 patients (18.8%) with abnormal Ccr, 31 (15.7%) with abnormal eGFR3, and 40 (20.3%) with abnormal eGFR5. Although there were no significant differences in the post Cr rate between patients with normal and abnormal Ccr, eGFR3 and eGFR5 values, the post Cr level was significantly higher in patients with eGFR3 and eGFR5 abnormality than in normal patients (P < 0.0001). Post Cr level tended to be higher in patients with Ccr abnormality (P = 0.0936 and P = 0.0875, respectively).

CONCLUSION: eGFR5 and the simpler eGFR3, rather than Ccr, are recommended as a preoperative renal function test in patients undergoing hepatectomy.

Key words: Estimated glomerular filtration rate; Creatinine clearance test; Hepatectomy; Renal function test

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INTRODUCTION

The outcome of hepatic resection has improved dramatically during the last 20 years, along with improvements in surgical techniques and perioperative management[1]. Operative mortality is now reportedly less than 1% at most institutions in Japan[2]. However, hepatectomy is associated with intraoperative blood loss, and postoperative complications such as liver failure, infection, bile leakage, ascites, and pleural effusion[3]. Uncontrolled ascites, pleural effusion and intraoperative blood loss disturb blood circulation, leading to dysfunction of not only the liver but also the kidney[4]. Therefore, for appropriate patient selection, it is necessary to evaluate preoperative liver and renal function accurately.

Glomerular filtration rate (GFR) is the most important and comprehensive index of renal function. GFR is measured by inulin clearance, but this takes > 2 h, and requires repeat collection of blood and urine every 15 min[5]. GFR is rarely measured in a clinical setting because of its intricacy. On the other hand, creatinine clearance (Ccr) has been measured clinically by a simple

method as a preoperative renal function test[6,7]. Although Ccr yields an approximate value for GFR, it is usually higher than the GFR as a result of secretion of 10%-15% of the creatinine into urine in the uriniferous tubule[8].

In 1999, Levey *et al* first reported a prediction equation known as modification of diet in renal disease (MDRD) for estimation of GFR on the basis of age, sex, race, serum creatinine, albumin and blood urea nitrogen (BUN) level for individuals of caucasian and black ethnicity[8]. MDRD has now been accepted as a standard method for evaluation of renal function in North America and Europe. This was a breakthrough for estimation of GFR because of its simplicity and ease of calculation. As a result of differences in physique between caucasian and black individuals, unique variables for estimating GFR in Japanese subjects have been investigated[9]. For this purpose, in 2008, two new equations for estimated GFR (eGFR) were devised on the basis of multiple regression analysis from inulin clearance data of 763 Japanese patients with chronic kidney disease and healthy controls[10]. These were: the three-variable equation for eGFR (mL/min per 1.73 m^2) = $194Cr^{-1.094} \times Age^{-0.287}$ (× 0.739; if the patient is female); the five-variable equation for eGFR (mL/min per 1.73 m^2) = $142Cr^{-0.923} \times Age^{-0.185} \times Alb^{0.414} \times BUN^{-0.233}$ (× 0.772; if the patient is female).

However, no studies have evaluated the usefulness of eGFR as a preoperative renal function test parameter. If eGFR is superior to Ccr as a preoperative renal function test, then eGFR should replace Ccr because of its simplicity of measurement. In this study, we retrospectively calculated the preoperative three-variable and five-variable equations for eGFR, and compared the results with Ccr, to clarify their superiority as a preoperative renal function test in patients undergoing hepatectomy.

MATERIALS AND METHODS

Patients

At Dokkyo Medical University, a total of 211 hepatic resections were performed for hepatobiliary disease between August 2006 and August 2008. Of these patients, 14 who were on hemodialysis, or for whom the results of preoperative Ccr were not available, were excluded. A total of 197 patients who underwent hepatectomy alone or hepatectomy plus combined surgery such as splenectomy, Hassab's operation, gastrectomy and colectomy for hepatocellular carcinoma (HCC), metastatic liver tumor, biliary malignancy and other benign disease were included in this study. There were 147 men and 50 women, with a mean age of 65.0 ± 10.0 years.

Measurements

Preoperative Ccr was measured by the 24-h method in all patients. The indocyanine green retention rate at 15 min (ICGR15) was also performed before hepatectomy. Serum creatinine, BUN and albumin levels were examined before hepatectomy, and 1, 2, 3, 5, 7, 14, 21 and 28 d after hepatectomy. The preoperative three-variable equation for eGFR (eGFR3) and the five-variable equation for eGFR (eGFR5) were calculated using the new formulas for Japanese patients[10]. The maximum serum creatinine and BUN levels after hepatectomy (post Cr, post BUN) were determined, and the postoperative rate of increase in the serum creatinine level (post Cr rate) was calculated by the following formula using the preoperative serum creatinine level (pre Cr) and post Cr. Post Cr rate (%) = (post Cr - preCr) × 100/post Cr.

Abnormal Ccr was defined as < 50 mL/min according to the New York Heart Association criteria[11,12], and groups with abnormal eGFR3 and eGFR5 were defined as < 60 mL/min per 1.73 m^2 according to the stage of chronic kidney disease[13].

Surgical procedures

Indications for hepatectomy were determined using the criteria of Makuuchi[14]. Portal embolization (PE) before hepatectomy was indicated in patients undergoing major hepatectomy when the estimated liver volume after hepatectomy was not sufficient to tolerate surgery (remnant liver volume < 40%)[15,16]. Transcatheter arterial embolization (TAE) was performed preoperatively in patients with massive HCC in order to occlude arterio-portal shunts. Preoperative biliary drainage was carried out in patients with obstructive jaundice. Liver resection was performed when the serum total bilirubin level was < 2.0 mg/dL. Simultaneous hepatectomy plus splenectomy, or Hassab's operation, were indicated for control of portal hypertension, esophageal and gastric varices, and thrombocytopenia (platelet count < $5.0 \times 10^4/mm^3$) in patients with HCC and liver cirrhosis[17]. The liver parenchyma was transected by the crush method using a Pean forceps or Cavitron Ultrasonic Aspirator while employing the intermittent Pringle maneuver. After resection of the liver tumors and subsequent hemostasis, the cut liver surface was coated with fibrin glue. The abdomen was then closed after placing drains around the cut liver surface. Major hepatectomy was classified as removal of one Couinaud segment or more, and minor hepatectomy as removal of less than one Couinaud segment.

Statistical analysis

Data were expressed as median (range). Non-parametric data were evaluated by χ^2 test and Kruskal-Wallis test between groups showing normal and abnormal values of Ccr, eGFR3 and eGFR5. Parametric data including post Cr and post Cr rate were compared among groups with normal and abnormal Ccr, eGFR3 and eGFR5 values using the Mann-Whitney *U* test. Correlations between Ccr or post Cr and eGFR3 and eGFR5 were analyzed using Pearson's correlation coefficient. Differences at $P < 0.05$ were considered to be significant.

RESULTS

Liver resections were performed for malignant disease in 180 patients and for benign disease in 17 patients. Malignant disease included 117 HCCs, 40 metastatic liver tumors, 16 biliary malignancies, three

Table 1 Indications for hepatectomy in 197 patients

	Overall	Abnormal Ccr group	Abnormal eGFR3 group	Abnormal eGFR5 group
Malignant tumors				
HCC	117	23	15	23
Cholangiocarcinoma	3	1	0	0
Metastatic liver tumor	40	7	7	8
Hilar BDC	12	2	0	1
Gall bladder carcinoma	4	1	2	2
Combined HCC	3	1	1	1
GIST	1	1	1	1
Total	180	36	26	36
Benign diseases				
Hepatolithiasis	2	0	0	0
Hepatic cyst	3	0	2	2
Hemagioma	2	0	1	1
Biliary cyst adenoma	3	1	1	1
Cholecystitis	2	0	1	0
Regenerative nodule	2	0	0	0
Donor	3	0	0	0
Total	17	1	5	4
		$P = 0.9065$	$P = 0.4630$	$P = 0.3889$

HCC: Hepatocellular carcinoma; BDC: Biliary duct carcinoma; GIST: Gastrointestinal stromal tumor.

Table 2 Background characteristics on Ccr

	Normal Ccr group (n = 160)	Abnormal Ccr group (n = 37)	P
Age (yr)	66 (30-82)	71 (46-85)	0.0344
Sex (male:female)	116:44	31:6	0.1552
Height (cm)	160.6 (134.5-179.6)	162.4 (133.9-182.7)	0.4109
Weight (kg)	59.2 (33.0-95.6)	61.6 (44.5-93.0)	0.3543
Hepatitis virus (-:+)	76:84	15:22	0.4441
$ICGR_{15}$ (%)	13 (1-74)	14 (4-49)	0.9085
Preoperative treatment (-:+)	134:26	32:5	0.6804

Hepatitis virus indicates hepatitis B, C, B + C; Preoperative treatment indicates biliary drainage, TAE and portal vein embolization.

cholangiocarcinomas and four other malignancies. Benign lesions included three giant hepatic cysts, three biliary cyst adenomas, three donors of living-related liver transplantation, two cases of hepatolithiasis, two of massive hemangioma, two of cholecystitis, and two cases of regenerative nodules. There were no significant differences in diseases between the groups with normal and abnormal Ccr, eGFR3 and eGFR5 values (Table 1).

Clinical background characteristics of the Ccr, eGFR3 and eGFR5 groups are shown in Tables 2-4, respectively. The median ages of patients with abnormal Ccr and eGFR5 values were significantly greater than those of patients with normal values. There were no significant differences in sex, height, weight, viral infection, $ICGR_{15}$, or frequency of preoperative treatment between the groups with normal and abnormal Ccr, eGFR3 and eGFR5 values.

Thirty-seven patients (18.8%) had abnormal Ccr, 31 (15.7%) had abnormal eGFR3, and 40 (20.3%) had abnormal eGFR5 values. Preoperative serum Cr and BUN levels, Ccr, eGFR3 and eGFR5 in all the patients

Table 3 Background characteristics on eGFR3

	Normal eGFR3 group (n = 160)	Abnormal eGFR3 group (n = 37)	P
Age (yr)	66 (30- 82)	69 (48-85)	0.0887
Gender (male:female)	125:41	22:9	0.6108
Height (cm)	161.0 (133.9-182.7)	160.4 (143.2-173.0)	0.0511
Weight (kg)	59.2 (33.0-93.4)	60.1 (44.6-95.6)	0.1656
Hepatitis virus (-:+)	77:89	14:17	0.9001
$ICGR_{15}$ (%)	13 (3-74)	13 (1-31)	0.9085
Preoperative treatment (-:+)	138:28	28:3	0.3129

eGFR3: Estimating glomerular filtration rate calculated by 3 factors; $ICGR_{15}$: Indocyanine green retention rate at 15 min.

Table 4 Background characteristics on eGFR5

	Normal eGFR5 group (n = 157)	Abnormal eGFR5 group (n = 40)	P
Age (yr)	65 (30-82)	71 (55-85)	0.0003
Sex (male:female)	119:38	28:12	0.4521
Height (cm)	161.5 (133.9-182.7)	158.5 (138.0-173.0)	0.0751
Weight (kg)	59.7 (33.0-95.6)	58.4 (44.0-93.0)	0.5478
Hepatitis virus (-:+)	75:82	16:24	0.3788
$ICGR_{15}$ (%)	13 (3-74)	14 (1-31)	0.6363
Preoperative treatment (-:+)	130:27	36:4	0.2644

with abnormal parameters were significantly worse than those in all the normal patients. Although there were no differences in serum albumin levels between the groups that had normal and abnormal Ccr and eGFR3, the serum albumin level was significantly decreased only in the group with eGFR5 abnormality (Table 5). The correlation between Ccr and eGFR5 was stronger than that between Ccr and eGFR3 (Figure 1).

Surgical details of the patients are shown in Table 6. Seventy-three patients underwent extensive hepatectomy. Among these patients, 28 (14.2%) underwent extended lobectomy, and 19 (9.6%) underwent lobectomy. According to the Couinaud classification, 26 patients (13.2%) underwent bisegmentectomy, and 37 (18.8%) underwent segmentectomy. Eighty-seven patients (44.2%) underwent partial hepatectomy. Although intraoperative blood loss in the patients with eGFR5 abnormality was significantly greater than that of normal patients, there were no significant differences in other surgical background factors, such as operation time, Pringle time, and type of surgical treatment between patients who were normal and abnormal for Ccr, eGFR3 and eGFR5.

Although neither operative nor hospital deaths were recorded, three patients (1.52%) required hemodialysis after hepatectomy because of multiple organ failure (two cases) and enterocolitis. Hepatic failure occurred in two patients. Postoperative results are shown in Table 7. Post Cr and post BUN of patients with eGFR3 and eGFR5 abnormalities were significantly higher than in normal patients, but post Cr and post BUN in patients with Ccr abnormality were not significantly higher than those in

Table 5 Preoperative measurements

	sCr (mg/dL)	BUN (mg/dL)	sAlb (g/dL)	Ccr (mL/min)	eGFR3	eGFR5
Overall (n = 197)	0.73 (0.33-1.74)	13 (5-31)	3.3 (2.1-4.4)	76.8 (1.3-226.1)	77.6 (30.7-196.8)	73.9 (29.8-171.0)
Normal Ccr (n = 160)	0.73 (0.33-1.51)	13 (5-30)	3.3 (2.1-4.4)	86.3 (50.0-226.1)	78.3 (36.7-171.0)	75.1 (36.4-171.0)
	$P < 0.05$	$P < 0.05$	NS	$P < 0.05$	$P < 0.05$	$P < 0.05$
Abnormal Ccr (n = 37)	0.81 (0.35-1.74)	15 (6-31)	3.3 (2.5-4.0)	34.1 (1.3-49.9)	66.6 (30.7-196.8)	64.5 (29.8-144.6)
Normal eGFR3 (n = 166)	0.71 (0.33-1.04)	13 (5-31)	3.3 (2.1-4.4)	83.6 (1.3-226.1)	80.7 (60.2-196.8)	77.0 (45.9-171.0)
	$P < 0.05$	$P < 0.05$	NS	$P < 0.05$	$P < 0.05$	$P < 0.05$
Abnormal eGFR3 (n = 31)	1.05 (0.75-1.74)	17 (9-26)	3.2 (2.1-4.1)	52.4 (11.8-129.1)	52.4 (30.7-59.9)	48.7 (29.8-68.7)
Normal eGFR5 (n = 157)	0.70 (0.33-1.07)	12 (5-22)	3.4 (2.1-4.4)	84.2 (1.3-226.1)	81.0 (58.0-196.8)	78.3 (62.1-171.0)
	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$
Abnormal eGFR5 (n = 40)	0.97 (0.70-1.74)	18 (10-31)	3.1 (2.1-4.1)	53.6 (11.8-129.1)	55.4 (30.7-68.3)	52.6 (29.8-59.9)

Cr: Creatinine; sCr: Serum creatinine; sAlb: Serum albumine; NS: Not significant.

Table 6 Surgical details

	Operative times (min)	Blood loss (mL)	Pringle time (min)	Hepatectomy (minor:major)	Hepatectomy (alone:plus)
Overall (n = 197)	320 (117-806)	590 (0-12762)	42 (5-176)	124:73	160:37
Normal Ccr (n = 160)	320 (117-721)	573 (0-7240)	43 (5-176)	99:61	132:28
	NS	NS	NS	NS	NS
Abnormal Ccr (n = 37)	321 (180-806)	618 (114-12762)	35 (9-98)	25:12	28:9
Normal eGFR3 (n = 166)	323 (117-721)	553 (0-7240)	42 (5-176)	103:63	136:30
	NS	NS	NS	NS	NS
Abnormal eGFR3 (n = 31)	297 (168-806)	680 (126-12762)	46 (12-87)	21:10	24:7
Normal eGFR5 (n = 157)	325 (117-721)	551 (0-7240)	42 (5-176)	97:60	128:29
	NS	$P < 0.05$	NS	NS	NS
Abnormal eGFR5 (n = 40)	296 (142-806)	694 (126-12762)	44 (11-98)	27:13	32:8

Minor: < 1 segmentectomy; Major: ⩾ 1 segmentectomy; Alone: Only hepatectomy; Plus: Hepatectomy with combined surgery.

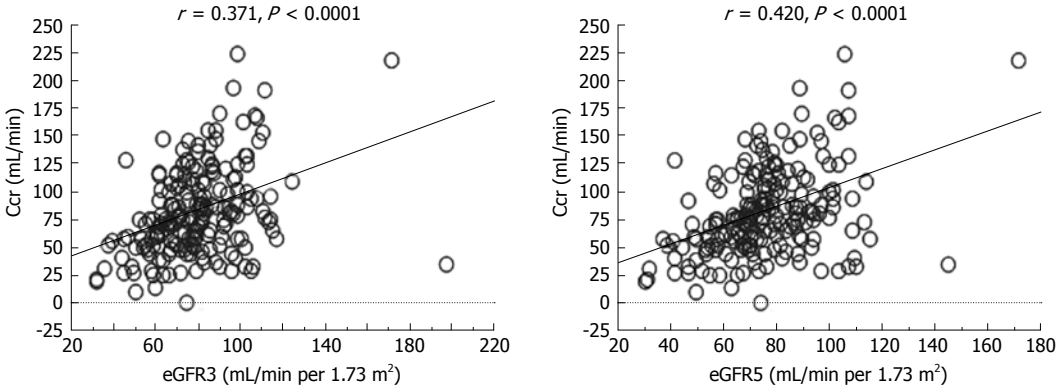

Figure 1 Relationship of measured Ccr to eGFR.

in normal patients.

Figure 2 shows the correlation between Ccr, eGFR3 and eGFR5 and post Cr. Although a weak correlation between post Cr and Ccr was observed, there were significant correlations between post Cr and eGFR5, and the correlation between post Cr and eGFR5 was higher than that between post Cr and eGFR3. Post Cr rates of patients with Ccr, eGFR3 and eGFR5 abnormality were not significant (Table 7).

DISCUSSION

As a preoperative renal function test, it is ideal to measure GFR by inulin clearance, but this is not practical in a clinical setting. Although the easiest renal function test to perform is measurement of serum creatinine level, use of this parameter alone is not recommended because it is affected by various factors such as muscle mass, sex, age, diet, and renal tubule function[9,13]. Therefore, Ccr has been measured routinely in patients undergoing major surgery for a long time. Determination of Ccr requires timed urine collection and blood sampling. Twenty-four-hour urine collection is especially inconvenient for patients with neurogenic bladder or the elderly. On the other hand, eGFR3 and eGFR5 require only a single blood sample, and can be estimated on the basis of age, sex, serum creatinine, BUN and albumin without the need for urine collection. If eGFR3

Table 7 Postoperative measurements

	Post Cr (mg/dL)	Post BUN (mg/dL)	Post Cr rate (%)
Overall (n = 197)	0.87 (0.43-8.43)	18 (7-97)	11.3 (0-88.1)
Normal Ccr (n = 160)	0.85 (0.43-5.71)	17 (7-81)	11.0 (0-88.1)
	P = 0.0936	P = 0.0875	P = 0.8253
Abnormal Ccr (n = 37)	0.95 (0.50-8.43)	20 (10-97)	12.3 (0-79.4)
Normal eGFR3 (n = 166)	0.83 (0.43-5.71)	17 (7-81)	11.4 (0-88.1)
	P < 0.0001	P = 0.0033	P = 0.4575
Abnormal eGFR3 (n = 31)	1.17 (0.70-8.43)	21 (11-97)	9.0 (0-79.4)
Normal eGFR5 (n = 157)	0.81 (0.43-5.71)	16 (7-81)	11.3 (0-88.1)
	P < 0.0001	P < 0.0001	P = 0.8950
Abnormal eGFR5 (n = 40)	1.14 (0.70-8.43)	23 (11-97)	11.5 (0-79.4)

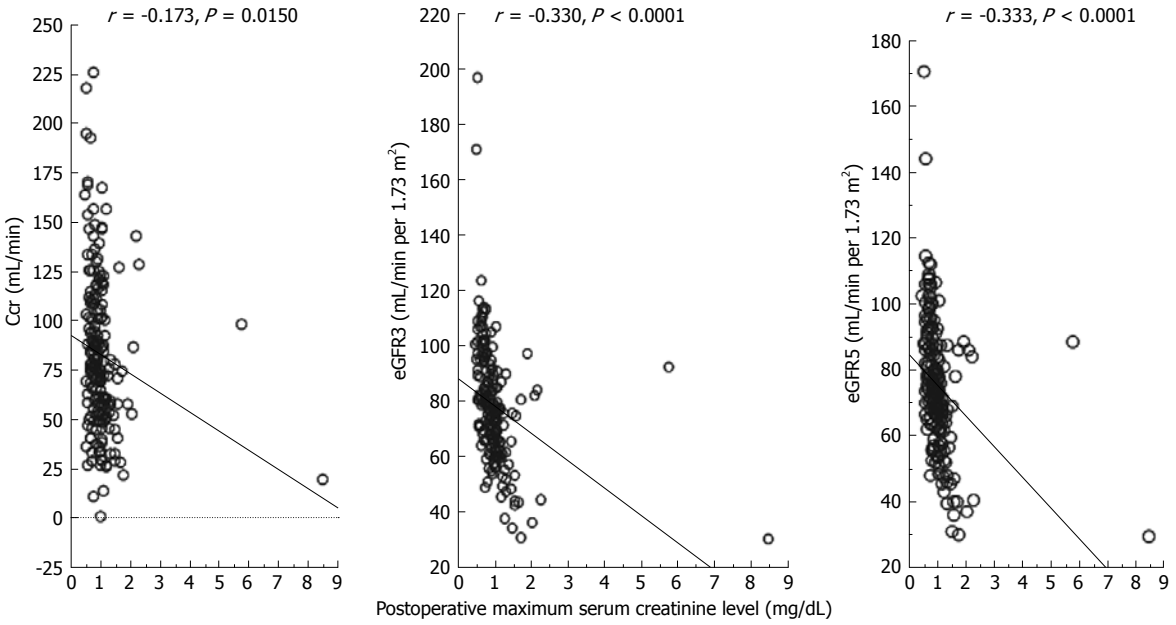

Figure 2 Relationship of the maximum increase in post Cr level to measured Ccr and eGFR.

and eGFR5 are superior to Ccr for preoperative renal function testing, there would be certain advantages in terms of clinical effort and cost.

The results of this study demonstrated that 24-h urine collection for measurement of Ccr no longer appears necessary on a routine basis for estimation of preoperative renal function. In fact, there were no significant differences in the post Cr or BUN level after hepatectomy between patients who had normal and abnormal preoperative Ccr values (Table 7). Post Cr and BUN levels in patients with eGFR3 and eGFR5 abnormalities were significantly higher than those in normal patients. In addition, the correlations between post Cr and eGFR3, and eGFR5 were significant, but that between post Cr and Ccr was not significant (Figure 2). These results indicate that eGFR3 and eGFR5 are superior to Ccr for predicting post renal dysfunction.

In this study, the post Cr rate after hepatectomy was also evaluated in patients who had normal and abnormal Ccr, eGFR3 and eGFR5 values, and no significant differences were evident (Table 7). We have already reported that hepatectomy can be performed safely without rapid and progressive deterioration of renal function in patients with non-uremic renal failure (Ccr of > 20 but < 50 mL/min)[6]. The factors affecting the post Cr rate after hepatectomy are preoperative liver function ($ICGR_{15}$ > 20%), intraoperative blood loss and operation time, and not preoperative renal dysfunction (data not shown).

In this study, eGFR5 differentiated 40 patients with preoperative renal dysfunction among 197 patients more sensitively than Ccr or eGFR3. There was a stronger positive correlation between Ccr and eGFR5 than between Ccr and eGFR3 (Figure 1). Although the equation for eGFR5 is a little complex, eGFR5 is more suitable than eGFR3 for patients undergoing hepatectomy. Since serum albumin level is one of the factors that reflects liver preservation, patients with HCC and liver cirrhosis frequently have lower levels of serum albumin. In fact, in this study, preoperative serum albumin levels ranged from 2.1 to 4.4 g/dL, with a median value of 3.3 g/dL. Thus, eGFR5 appears to be a more acceptable parameter for accurate preoperative evaluation of renal function in hepatectomy patients presenting a wide range of serum albumin levels.

To the best of our knowledge, this is the first retrospective study to have compared Ccr and eGFR as a preoperative renal function test in patients undergoing

hepatectomy. Since equations for eGFR in individuals of caucasian, black and Japanese ethnicity have been established, eGFR is now almost universally available. We suggest that eGFR3 and eGFR5 are useful as preoperative renal function parameters in patients undergoing hepatectomy worldwide.

In conclusion, we recommend eGFR5 using serum albumin level as a preoperative renal function test in patients undergoing hepatectomy. Ccr is no longer recommended as a first-choice preoperative renal function test.

COMMENTS

Background

Although creatinine clearance (Ccr) has been measured clinically by a simple method as a preoperative renal function test, Ccr is not strictly equal to glomerular filtration rate (GFR). Recently, an equation for estimated GFR (eGFR) for Japanese individuals has been postulated. It has been accepted that eGFR is equal to measured GFR in chronic kidney disease. However, there have been no previous studies regarding the reliability of eGFR as a preoperative renal function test.

Research frontiers

If eGFR is superior to, or equal to Ccr as a preoperative renal function test, eGFR should replace Ccr because of its simplicity of measurement. The authors retrospectively compared Ccr and eGFR as a preoperative renal function test in patients undergoing hepatectomy.

Innovations and breakthroughs

eGFR is useful as preoperative renal function parameters in patients undergoing hepatectomy. Ccr is no longer recommended as a first-choice preoperative renal function test.

Applications

Although Ccr has been used as preoperative renal function test, eGFR should replace Ccr as a routine preoperative renal function test in various surgical fields.

Terminology

eGFR is estimated GFR which is calculated from age, sex, serum creatinine value (eGFR3), or adding serum albumin concentration and BUN value (eGFR5).

Peer review

This is a well-written paper on normal and sensitive parameters of renal function, i.e. eGFR3 and eGFR5 as predictors of renal function after hepatectomy. These parameters seem easy to determine, accurate and well-associated with the stage of kidney disease. The study seems well-designed and performed, original in concept and statistically valid.

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BRIEF ARTICLES

Cancer stem cell markers CD133 and CD24 correlate with invasiveness and differentiation in colorectal adenocarcinoma

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Abstract

AIM: To verify that CD markers are available for detecting cancer stem cell populations and to evaluate their clinical significance in colon cancer.

METHODS: Immunohistochemistry for CD133, CD24 and CD44 was performed on the tissue microarray of 523 colorectal adenocarcinomas. Medical records were reviewed and clinicopathological analysis was performed.

RESULTS: In colorectal adenocarcinoma, 128 of 523 cases (24.5%) were positive and 395 cases (75.5%) were negative for CD133 expression. Two hundred and sixty-four of 523 cases (50.5%) were positive and 259 cases (49.5%) were negative for CD24 expression. Five hundred and two of 523 cases (96%) were negative and 21 cases (4%) were positive for CD44 expression. Upon clinicopathological analysis, CD133 expression was present more in male patients (P = 0.002) and in advanced T stage cancer (P = 0.024). Correlation between CD24 expression and clinicopathological factors was seen in the degree of differentiation (P = 0.006). Correlation between CD44 expression and clinicopathological factors was seen in the tumor size (P = 0.001). Survival was not significantly related to CD133, CD24 and CD44 expression.

CONCLUSION: CD markers were related to invasiveness and differentiation of colorectal adenocarcinoma. However, CD expression was not closely related to survival.

Key words: CD133; CD24; CD44; Colon cancer stem cells; Colorectal adenocarcinoma

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INTRODUCTION

Colorectal adenocarcinoma is the second most common type of cancer and a major cause of cancer-related morbidity and mortality in the Western world[1]. The incidence of colorectal cancer has increased from 5.8% in 1980 to 10.3% in 2000 in South Korea, in part because of Westernization of the diet[2].

Countless treatment protocols, including chemotherapy and radiation, have been applied to colorectal cancer and a number of studies have identified conventional prognostic factors[3]. However, a complete cure of colorectal cancer has not been accomplished despite numerous efforts. Recently, the prospective identification of colon cancer stem cells

has received major attention because of their potential for colon cancer treatment[4,5]. Current colon cancer treatment modalities target proliferating cells, but colon cancer stem cells are thought to be slowly cycling cells; therefore, they may escape present targeted interventions because they are not actively proliferating. This may be one of the most important reasons behind colon cancer treatment failure and recurrence. It is important to validate *in vitro/in vivo* colon cancer stem cell findings in clinical samples. This will be a critical step toward the development of effective targeted colon cancer treatment, but thus far, no data are available on the clinical implications of the suggested colon cancer stem cells in clinical samples.

Recently, several CD markers have been identified as solid cancer stem cell markers. CD133, also known as PROML1 or prominin, is a stem cell surface antigen that has been recently identified as a potential cancer stem cell marker in brain, colon and prostate cancer[4-7]. CD44, also known as homing cell adhesion molecule, is a cell surface glycoprotein expressed on lymphocytes, monocytes and granulocytes, which has been identified as a stem cell marker in breast and head and neck cancer[8,9]. CD24, a cell surface marker, is a single chain sialoglycoprotein with a molecular mass of 42 kDa. CD24- and CD44-expressing pancreatic cancer cells show cancer stem cell characteristics[10]. Here, we report the identification of CD133-, CD24- and CD44-positive tumor cells in colon tumor sections by an immunohistochemistry-based technique, and discuss the findings in conjunction with clinicopathological data.

MATERIALS AND METHODS

Patients and specimens

This retrospective study consisted of a consecutive series of 523 colorectal adenocarcinomas with complete histopathological data available. Patients were diagnosed and treated at the Hanyang University Hospital, Seoul, Korea, from January 1991 to August 2001. There were 295 male and 228 female patients, with ages ranging from 17 to 87 years (mean, 59.0 years). The adenocarcinomas were located in the cecum (n = 18), ascending colon (n = 77), hepatic flexure (n = 12), transverse colon (n = 26), splenic flexure (n = 4), descending colon (n = 24), sigmoid colon (n =112), and rectum (n = 250). Their sizes ranged from 0.3 to 15 cm (mean, 5.7 cm).

All tissue samples were formalin-fixed and paraffin-embedded. Hematoxylin and eosin (HE)-stained slides, pathological reports, and other medical records were reviewed to confirm the diagnosis and clinicopathological parameters, including age, gender, tumor location, tumor size, depth of invasion, lymph node metastasis, distant metastasis, American Joint Committee on Cancer (AJCC) stage, Dukes' stage, degree of differentiation, lymphovascular invasion and patient survival.

Tissue microarray (TMA) construction

The most representative area was carefully selected and marked on an H&E-stained slide. The TMA was assembled using a tissue-array instrument (AccuMac arrayer; ISU ABXIS Co. Ltd., Seoul, Korea) that consisted of thin-walled stainless steel punches and stylets used to empty and transfer the needle content. The assembly was held in an X-Y position guide equipped with semiautomatic micrometers, with a 1-mm increment between individual samples and a 3-mm punch depth stop device. Briefly, the instrument was used to create holes in a recipient block with defined array cores. A solid stylet, which closely fits the needle, was used to transfer the tissue cores into the recipient block. Taking into account the limitations of the representative areas of the tumor, we used triplicate 1-mm diameter tissue cores from each donor block.

HE and immunohistochemical staining

Multiple 4-μm sections were cut with a Leica microtome. Sections were transferred to adhesive-coated slides. One section was routinely deparaffinized with standard xylene and hydrated through graded ethanol in water, stained with HE, and covered with a coverslip. For immunohistochemical staining, the TMA slides were dewaxed by heating at 55℃ for 30 min and by three 5-min washes with xylene. Tissues were rehydrated by a series of 5-min washes in 100%, 90% and 70% ethanol and phosphate buffered saline (PBS). Antigen retrieval was performed by microwaving the samples for 4 min 20 s at full power in 250 mL 10 mmol/L sodium citrate (pH 6.0). Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxidase for 20 min. The primary polyclonal rabbit anti-CD133 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was diluted 1:200 using goat serum and incubated at room temperature for 1 h. The primary monoclonal mouse anti-CD24 antibody (Santa Cruz Biotechnology) was diluted 1:50 and the primary monoclonal mouse anti-CD44s antibody (Neomarkers, CA, USA) was diluted 1:100. After three 2-min washes with PBS, the sections were incubated with a biotinylated goat secondary antibody for 30 min (DAKO, Carpinteria, CA, USA). After three 2-min washes with PBS, horseradish peroxidase-streptoavidin (DAKO) was added to the section for 30 min, followed by another three 2-min washes with PBS. The samples were developed with 3,3'-diaminobenzidine substrate (Vector Laboratories, Burlington, Ontario, Canada) for 1 min and counterstained with Mayer's hematoxylin. The slides were dehydrated following a standard procedure and sealed with coverslips. We used the glioblastoma tissue as a positive control of CD133, the tonsillar lymphoid tissue as a positive control of CD24, and the tonsillar mucosal epithelial tissue as a positive control of CD44. Negative controls were performed by omitting CD133, CD24 and CD44 antibodies during the primary antibody incubation. The representative sections of CD133, CD24 and CD44 immunostaining are shown in Figure 1.

Interpretation of CD133, CD24 and CD44 expression

CD133, CD24 and CD44 expression was evaluated

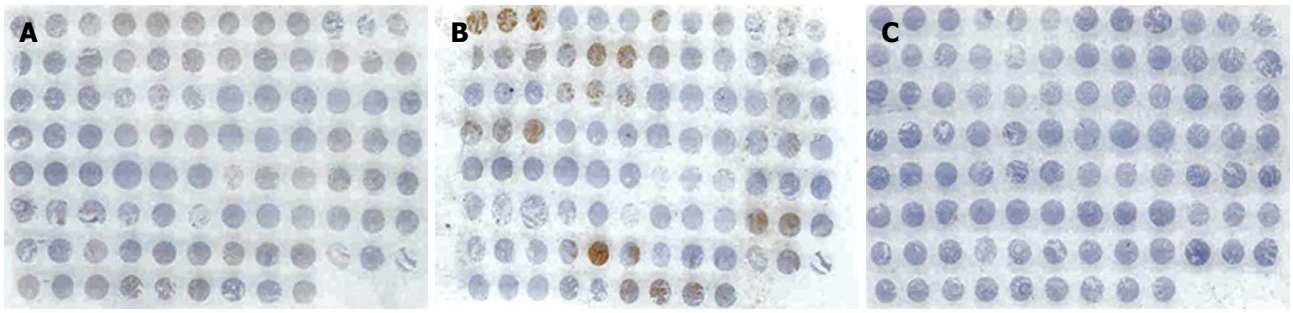

Figure 1 Representative photograph of the TMA slides with immunohistochemical staining. A: CD133; B: CD24; C: CD44.

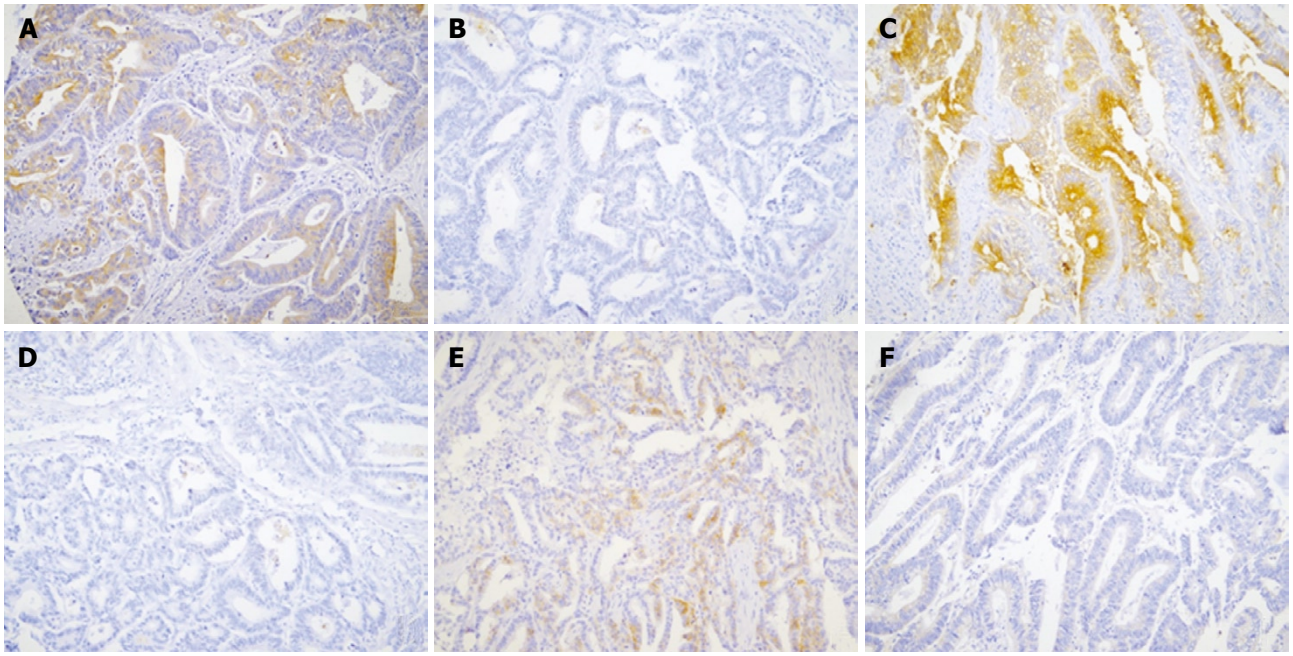

Figure 2 Representative photographs of CD marker expression in colorectal adenocarcinoma. Positive staining (A) and negative staining (B) for CD133. Positive staining (C) and negative staining (D) for CD24. Positive staining (E) and negative staining (F) for CD44.

semi-quantitatively by two independent pathologists (Paik SS and Song YS), in a blinded fashion without knowledge of clinical and pathological information. The sections were scanned at high magnification to assess the positivity of staining in tumor cells. We regarded the staining as positive in cases with cytoplasmic positivity. In cases of discrepant assessments, slides were reinvestigated by both pathologists under a multi-head microscope and an agreement was obtained.

Statistical analysis

Statistical analysis was performed using SPSS version 12.0 software (SPSS, Chicago, IL, USA). The χ^2 test was used to examine the association between CD133, CD24 and CD44 expression and various clinicopathological characteristics, including age, gender, tumor location, tumor size, TNM category, AJCC stage, Dukes' stage, degree of differentiation, and lymphovascular invasion. The Kaplan-Meier method was used to calculate overall survival curves. Univariate survival analysis with the log-rank test was used to compare the difference between the survival rates of the patients' subgroups. Multivariate survival analysis with Cox's proportional hazard regression model was used to evaluate the independent prognostic factors. A difference of $P < 0.05$ between groups was considered significant.

RESULTS

Pattern of CD marker expression in colorectal adenocarcinoma

CD marker expression was evaluated in colorectal adenocarcinoma. One hundred and twenty-eight of 523 cases (24.5%) were positive and 395 cases (75.5%) were negative for CD133 expression (Figure 2A and B). Two hundred and sixty-four of 523 cases (50.5%) were positive and 259 cases (49.5%) were negative for CD24 expression (Figure 2C and D). However, 21 of 523 cases (4%) were positive and 502 cases (96%) were negative for CD44 expression (Figure 2E and F).

Correlation of CD marker expression and clinicopathological parameters in colorectal adenocarcinoma

Upon clinicopathological analysis, CD133 expression

Table 1 Correlation between CD133 and CD24 expression and clinicopathological factors in colorectal cancer (*n* = 523)

	n	Expression of CD133		*P* value	Expression of CD24		*P* value
		Negative (*n* = 395)	Positive (*n* = 128)	(χ^2-test)	Negative (*n* = 259)	Positive (*n* = 264)	(χ^2-test)
Age (yr)				0.431			0.999
< 59	261	201	60		129	132	
⩾ 59	262	194	68		130	132	
Gender				0.002			0.261
Male	295	208	87		140	155	
Female	228	187	41		120	108	
Tumor location				0.735			0.315
Right colon	133	99	34		71	62	
Left colon	390	296	94		189	201	
Tumor size				0.436			0.658
< 5.5 cm	254	188	66		124	130	
⩾ 5.5 cm	269	207	62		136	133	
T category				0.024[1]			0.219
Tis	12	12	0		8	4	
T1	9	8	1		4	5	
T2	37	29	8		20	17	
T3	452	337	115		223	229	
T4	13	9	4		5	8	
N category				0.890			0.525
N0	234	178	56		113	121	
N1	132	96	36		65	67	
N2	157	121	36		81	76	
M category				0.555			0.482
M0	502	378	124		248	254	
M1	21	17	4		12	9	
AJCC stage				0.259			0.998
0	12	12	0		8	4	
I	34	29	5		18	16	
ⅡA, ⅡB	185	134	51		85	100	
ⅢA, ⅢB, ⅢC	271	203	68		137	134	
Ⅳ	21	17	4		12	9	
Dukes' stage				0.560			0.515
A	17	16	1		10	7	
B1, B2	210	157	53		98	112	
C1, C2	275	205	70		139	136	
D	21	17	4		12	9	
Degree of differentiation				0.084			0.006[1]
Well	23	16	7		10	13	
Moderate	393	300	93		183	210	
Poorly	100	74	26		61	39	
Undifferentiated	7	5	2		5	2	
Lymphatic invasion				0.848			0.772
Absent	225	169	56		110	115	
Present	298	226	72		150	148	
Vascular invasion				0.740			0.508
Absent	513	387	126		254	259	
Present	10	8	2		6	4	

[1]χ^2 test for linear trend. AJCC: American Joint Committee on Cancer; CD: Cluster of differentiation.

was present more in male patients (P = 0.002) and in advanced T stage cancer (P = 0.024) (Table 1). Correlation between CD24 expression and clinicopathological factors was seen in the degree of differentiation (P = 0.006) (Table 1). Correlation between CD44 expression and clinicopathological factors was seen for the tumor size (P = 0.001) (data not shown).

Correlation of CD marker expression and patient overall survival

We examined the effect of CD marker expression on clinicopathological prognostic factors in colorectal adenocarcinoma. A significant prognostic influence of age, histological grade, AJCC stage, lymphatic invasion and vascular invasion on overall survival was found by univariate and/or multivariate analyses. However, no impact of CD133, CD24 and CD44 expression on overall survival was observed in univariate and multivariate survival analyses. Kaplan-Meier survival curves and log-rank tests showed no significant correlation between patient survival and CD133, CD24 and CD44 expression (P = 0.774, P = 0.775 and P = 0.636, respectively) (Figure 3).

DISCUSSION

Cancer stem cells have recently been proposed to

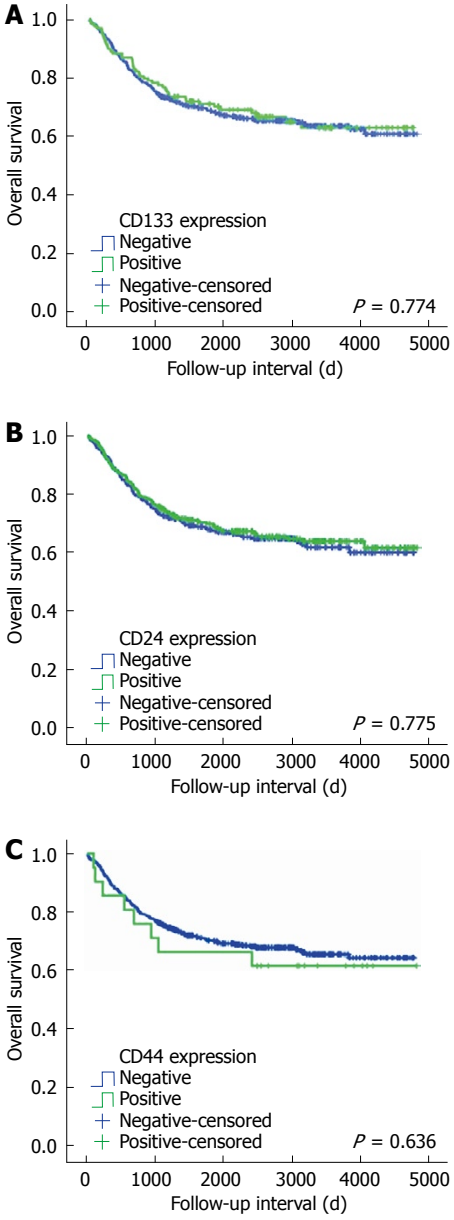

Figure 3 Cumulative survivals according to CD133 (*P* = 0.774) (A), CD24 (*P* = 0.775) (B) and CD44 (*P* = 0.636) (C) expression in colorectal cancer patients (Kaplan-Meier method).

be the cancer initiating cells that are responsible for tumorigenesis and for contributing to cancer resistance in leukemia[11]. Compared to leukemia, evidence for the existence of cancer stem cells in solid tumors has been more difficult to obtain for several reasons. Cells within solid tumors are less accessible, and functional assays suitable for detecting and quantifying normal stem cells from many organs have not yet been developed, and the cell surface markers required to isolate such cells have not been identified fully. However, there have been some impressive studies in this area recently. Advances have been made in identifying and enriching cancer stem cells in several solid tumors, including breast, brain and colon cancers[4-6,8-10].

Lapidot *et al*[12] have shown that leukemia-initiating stem cells present in the peripheral blood of acute myelogenous leukemia (AML) patients can induce AML when transplanted into severe combined immunodeficient mice. In 2003, Al-Hajj *et al*[8] isolated human breast cancer stem cells that can cause breast cancer in non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice through serial transplantations, which suggests a capacity for self-renewal. The following year, Singh *et al*[6] have found evidence of stem cell involvement in brain cancer. Recently, O'Brien *et al*[4] have demonstrated that CD133-positive colon-cancer-initiating cells in the human colon cancer specimen generate tumors in the renal capsule of pre-irradiated NOD/SCID mice. More recently, cells have been isolated from human prostate cancer patients, which can produce serially transplantable prostate tumors in NOD/SCID mice[10]. Even though definitive cancer stem cell markers have not been found in all the previously mentioned studies, these studies have revealed that only a small subset of cells in different tumor types is capable of tumor formation and several candidate stem cell markers have been evaluated. While CD133, CD24 and CD44 have been tested as cancer stem cell markers for serial transplantation studies in various cancers, their prognostic value has not been elucidated clearly[4-10].

CD133, which is one of the most important cancer stem cell markers[4-7], was stained fairly well in 24.5% of our colon cancer patients. In terms of clinicopathological parameters, CD133 expression was related to gender and T stage. The present study revealed that male gender was positively related to CD133 expression. T0 and T1 colon cancers showed lower incidence of CD133 protein expression compared to advanced colon cancer.

CD24, another important cancer stem cell marker, was expressed in 50.5% of the colon cancer patients. Correlation between CD24 expression and clinicopathological factors was seen in degree of differentiation. CD24 consists of a small protein core comprising 27 amino acids, which is extensively glycosylated and is bound to the membrane via a phosphatidylinositol anchor[13-15]. Several reports have shown that CD24 can be expressed on several solid tumors such as small cell lung cancer, neuroblastoma, rhabdomyosarcoma and renal cell cancer[16,17]. Lim *et al*[18] have reported that CD24 expression is related to lymph node metastasis in colon cancer. Weichert *et al*[19] have shown that cytoplasmic CD24 expression in colorectal cancer is independently correlated with shortened patient survival. We have not seen any positive correlation between CD24 expression and nodal status and patient survival.

CD44 is an unique adhesion molecule and several studies have revealed that CD44 is overexpressed at the mRNA and protein levels in colon cancer[20,21]. In our study, large tumors bigger than 5.5 cm showed more frequent CD44 expression, which indicated that CD44 expression was related to clinical tumor burden.

Our results in human colon cancer specimens showed various expression patterns of CD markers. This is believed to be the first trial to verify the

relationship between well-known prognostic factors of colon cancer and conventional cancer stem cell markers. Tumor invasiveness and differentiation were identified as clinicopathological factors related to cancer stem cell markers, especially CD133 and CD24.

For further study, other colon stem cell markers which are related to patient survival should be found for clinical application of colon cancer stem cells. Several studies have indicated that pleuripotency-related factors, including Oct3/4, are related to cancer development[22-24]. Beside CD markers, other cancer stem cell markers may help distinguish cancer stem cells from cancer cells.

In summary, we have presented some evidence that several conventional clinical factors were related to cancer stem cell markers in colorectal adenocarcinoma. However, CD133, CD24 and CD44 expression did not show a close relationship with the survival outcome of colorectal adenocarcinoma. These results warrant further careful and well-designed studies of colon cancer stem cells as markers for clinical application.

COMMENTS

Background

Colorectal adenocarcinoma is the second most common type of cancer and a major cause of cancer-related morbidity and mortality in the Western world. Countless treatment protocols including chemotherapy and radiation have been applied to colorectal cancer treatment and a number of studies have identified conventional prognostic factors. Recently, the prospective identification of colon cancer stem cells has received major attention because of their potential for colon cancer treatment.

Research frontiers

This study focused on the identification of CD133-, CD24- and CD44-positive tumor cells in colon tumor sections by an immunohistochemistry-based technique, and the findings are discussed in conjunction with clinicopathological data.

Innovations and breakthroughs

Results in human colon cancer specimens showed various expression patterns of CD markers. This is believed to be the first trial for verifying the relationship between well-known prognostic factors of colon cancer and cancer stem cell markers. Tumor invasiveness and differentiation were identified as clinicopathological factors related to cancer stem cell markers.

Applications

The authors have presented some evidence that several conventional clinical factors were related to cancer stem cell markers in colorectal adenocarcinoma. However, CD133, CD24 and CD44 expression did not show a close relationship with survival. These results warrant further careful and well-designed studies of colon cancer stem cells as markers for clinical application.

Terminology

CD133, also known as PROML1 or prominin, is a stem cell surface antigen that has been identified recently as a potential cancer stem cell marker in brain, colon and prostate cancer. CD44, also known as homing cell adhesion molecule, is a cell surface glycoprotein expressed on lymphocytes, monocytes and granulocytes, which has been identified as a stem cell marker in breast and head and neck cancer. CD24, a cell surface marker, is a single chain sialoglycoprotein with a molecular mass of 42 kDa.

Peer review

The authors have presented some evidence that several conventional clinical factors were related to cancer stem cell markers in colorectal adenocarcinoma. This study is modestly reported with respect to a careful and large study. The approach needs to be encouraged, despite the relatively negative nature of the study with respect to the utility of the markers as a prognostic markers.

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How good is cola for dissolution of gastric phytobezoars?

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Abstract

AIM: To evaluate the efficacy of cola treatment for gastric phytobezoars, including diospyrobezoars.

METHODS: A total of 17 patients (range: 48 to 78 years) with symptomatic gastric phytobezoars treated with cola and adjuvant endoscopic therapy were reviewed. Three liters of cola lavage (10 cases) or drink (7 cases) were initially used, and then endoscopic fragmentation was done for the remnant bezoars by using a lithotripsy basket or a polypectomy snare. The overall success of dissolving a gastric phytobezoars with using three liters of cola and the clinical and endoscopic findings were compared retrospectively between four cases of complete dissolution by using only cola and 13 cases of partial dissolution with cola.

RESULTS: After 3 L of cola lavage or drinking, a complete dissolution of bezoars was achieved in four patients (23.5%), while 13 cases (76.5%) were only partially dissolved. Phytobezoars (4 of 6 cases) were observed more frequently than diospyrobezoars (0 of 11) in the group that underwent complete dissolution (P = 0.006). Gender, symptom duration, size of bezoar and method of cola administration were not significantly different between the two groups. Twelve of 13 patients with residual bezoars were completely treated with a combination of cola and endoscopic fragmentation.

CONCLUSION: The rate of complete dissolution with three liters of cola was 23.5%, but no case of diospyrobezoar was completely dissolved using this method. However, pretreatment with cola may be helpful and facilitate endoscopic fragmentation of gastric phytobezoars.

Key words: Gastric phytobezoars; Diospyrobezoars; Cola; Dissolution; Clinical efficacy

Peer reviewer: Radha K Dhiman, Associate Professor, Department of Hepatology, Postgraduate Institute of Medical Education and Research, Chandigarh 160012, India

Lee BJ, Park JJ, Chun HJ, Kim JH, Yeon JE, Jeen YT, Kim JS, Byun KS, Lee SW, Choi JH, Kim CD, Ryu HS, Bak YT. How good is cola for dissolution of gastric phytobezoars? *World J Gastroenterol* 2009; 15(18): 2265-2269 Available from: URL: http://www.wjgnet.com/1007-9327/15/2265.asp DOI: http://dx.doi.org/10.3748/wjg.15.2265

INTRODUCTION

Bezoars are hard masses or concretions of indigestible food, vegetable fiber or hair that are found in the gastrointestinal (GI) tract, usually in the stomach. Although the incidence of bezoars is unknown, the reported incidence is about 0.4%[1]. Bezoars are usually classified into four types according to their composition: phytobezoars, trichobezoars, medication bezoars and lactobezoars. Dissolution therapy with proteolytic or cellulase enzymes[2], endoscopic fragmentation or aspiration and surgery have been proposed as the treatment options for bezoars, and these treatments have a wide range of efficacy[3]. Ingestion of persimmons is considered the most common cause of phytobezoars in some countries[4,5]. Because of their hard consistency, endoscopic therapy with fragmentation or enzymatic dissolution is challenging and sometimes mechanical fragmentation cannot be accomplished.

Recently, dissolution bezoars with cola has been described to be one of the effective treatment options for the treatment of gastric phytobezoars. However,

Table 1 Basal characteristics of 17 consecutive patients

Case no.	Gender	Age	Symptoms	Duration (d)	Comorbidity	Type of bezoar	Endoscopic findings	Size of bezoar
CD group								
1	F	69	Indigestion	21	None	Phytobezoar	GU	Above 50%
2	F	62	Epigastric soreness	21	DM	Phytobezoar	GU	Above 50%
3	F	49	Indigestion, epigastric soreness	14	DM	Phytobezoar	Pyloric stenosis	Below 50%
4	F	51	Indigestion	20	DM	Phytobezoar	GU	Below 50%
PD group								
5	M	48	Epigastric soreness	8	None	Phytobezoar	DU scar with stenosis	Below 50%
6	F	57	Indigestion	21	None	Diospyrobezoar	Pyloric stenosis	Above 50%
7	M	71	Indigestion	90	None	Diospyrobezoar	Pyloric stenosis	Below 50%
8	F	65	Epigastric soreness	10	HTN	Diospyrobezoar	Pyloric stenosis	Above 50%
9	F	61	Indigestion	14	DM	Diospyrobezoar	Pyloric stenosis	Below 50%
10	F	57	Indigestion	21	None	Diospyrobezoar	Pyloric stenosis	Below 50%
11	F	67	Epigastric soreness	60	DM	Diospyrobezoar	GU	Below 50%
12	M	63	Epigastric soreness	21	HTN	Phytobezoar	Pyloric stenosis	Above 50%
13	M	78	Indigestion	30	DM	Diospyrobezoar	GU	Above 50%
14	F	75	Epigastric soreness	90	DM	Diospyrobezoar	None	Below 50%
15	F	54	None	7	HTN	Diospyrobezoar	GU	Below 50%
16	F	61	Nausea and vomiting	7	None	Diospyrobezoar	GU	Below 50%
17	M	63	Epigastric soreness	30	None	Diospyrobezoar	Pyloric stenosis	Above 50%

GU: Gastric ulcer; DM: Diabetes mellitus; CD: Complete dissolution; PD: Partial dissolution.

only commentaries on individual cases or small number of cases have been reported on the cola dissolution of phytobezoars[6-12]. To elucidate the efficacy of cola for dissolving phytobezoars, further evaluations with large number cases are needed. Here, we report the clinical results of 17 consecutive patients with gastric phytobezoars, including diospyrobezoars, who were initially treated by three liters of cola gastric lavage or drink.

MATERIALS AND METHODS

The medical records of 17 patients with gastric phytobezoars treated by cola gastric lavage at Korea University Hospital, Seoul, between 2002 and 2008 were retrospectively reviewed. Diagnosis and classification of gastric bezoars were based upon clinical and endoscopic findings. Diospyrobezoar was diagnosed by history of eating persimmons and the endoscopic typical characteristics such as cemented seeds of persimmon or hard consistency or darkish-brown color. The size of each bezoar was grossly estimated as occupied percentage of the lumen of the stomach. The patients' median age was 63 years (range: 48-78). Seven patients had a long-standing history of diabetes mellitus (DM), four had a history of arterial hypertension (AHT) and the other patients had no significant past or concurrent diseases. On the initial endoscopic evaluation, 11 cases had darkish-brown colored diospyrobezoar and 6 cases had bright brown or yellow colored phytobezoars (Table 1). After diagnosing the bezoar, two nasogastric tubes (16 F) were placed for performing gastric cola lavage. One tube was used for the continuous administration of cola, and the other for natural drainage. A lavage with three liters of cola was performed over 12 h. All the procedures were done after obtaining informed consent from all the patients. Among the 17 patients, 8 patients drank the three liters of cola as they had refused to have the nasogastric tube inserted (Table 2). The nine patients with nasogastric tubes were kept in the recumbent position to prevent aspiration. The patients with DM had blood glucose monitoring by a glucometer. One day after the lavage with three liters of cola (or after drinking the same amount of cola), second-look endoscopic examinations were performed for assessing the bezoar. The status of the bezoars was then described, i.e. whether or not the bezoar remained in place, and whether it had changed its size and consistency. When the bezoar had disappeared, we considered this as complete dissolution. When it had a decreased size and a softened consistency by palpation with the biopsy forcep, then the response was considered as partial dissolution. If no change of the size and consistency was observed, then this was considered as lack of response. As the result of the initial administration of cola, patients were divided into two groups, i.e with either complete dissolution or partial dissolution. The clinical and endoscopic features and the method of cola administration were then retrospectively compared between two groups.

The endoscope we used was an Olympus GIF Q 260 or a H 260 (Olympus, Tokyo, Japan). If a residual bezoar was observed, then endoscopic fragmentation was done using a lithotripsy basket (GML-90-26-180, Medi-Globe, Achenmuhle, Germany) or a polypectomy snare (SD-5L-1, Olympus, Japan) (Figure 1A and B). Finally, fragmented bezoars were crushed and retrieved with a biopsy forcep or a pentagon grasping forcep (Figure 1C and D). After the procedures, all the patients were advised to drink an additional 3 L of cola. Re-examination endoscopy was done one day after the procedures. All the endoscopic procedures were performed by three expert endoscopists.

Fisher exact test and the Mann-whitney *U* test were used for the comparison of clinical, endoscopic, and method of administration of cola. $P < 0.05$ were regarded as statistically significant.

RESULTS

After lavage with (or drinking) 3 L of cola, a complete

Table 2 Results of cola administration and endoscopic intervention

Case no.	Initial method of cola administration	Methods of endoscopic treatment	Endoscopic procedure time (min)	No. of endoscopic sessions (*n*)	No. of used instruments (*n*)	Total amount of administered cola (liters)	Hospital stay (d)
CD group							
1	3 L drink			0	0	3	2
2	3 L lavage			0	0	3	2
3	3 L lavage			0	0	3	3
4	3 L lavage			0	0	3	2
PD group							
5	3 L drink	Mechanical lithotripsy	8	1	1	6	3
6	3 L lavage	Mechanical lithotripsy	45	1	1	6	3
7	3 L drink	Mechanical lithotripsy	14	1	2	6	2
8	3 L lavage	Mechanical lithotripsy	44	1	1	6	3
9	3 L drink	Mechanical lithotripsy	62	1	2	6	4
10	3 L lavage	Mechanical lithotripsy	42	1	1	6	3
11	3 L lavage	Mechanical lithotripsy	50	1	1	6	7
12	3 L lavage	Mechanical lithotripsy	30	1	1	6	3
13	3 L lavage	Polypectomy snare	52	2	2	9	3
14	3 L drink	Polypectomy snare	40	2	3	6	5
15	3 L drink	Polypectomy snare	58	2	3	9	5
16	3 L drink	Polypectomy snare	62	1	4	6	5
17	3 L drink	Polypectomy snare	72	2	5	6	3

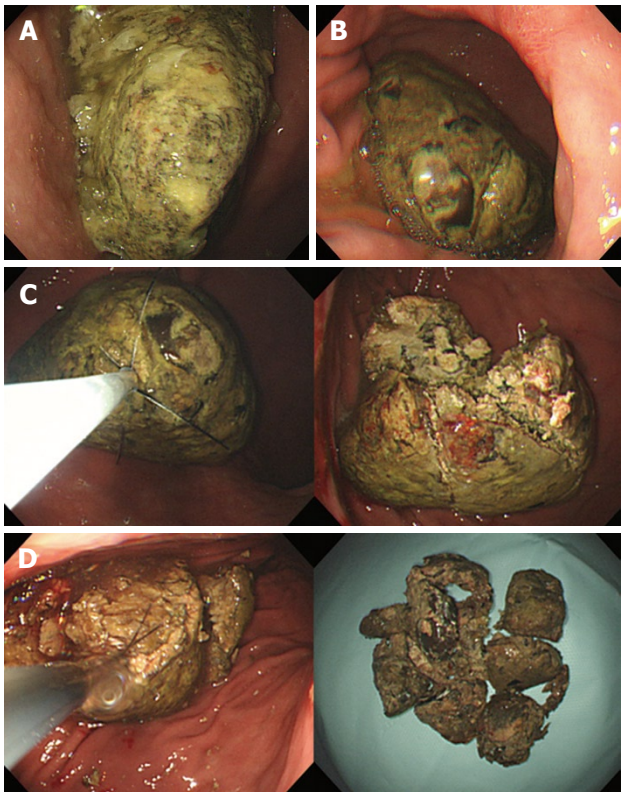

Figure 1 Endoscopic views of diospyrobezoar. A: Initial endoscopic view. A huge dark brownish-colored diospyrobezoar was noted in the stomach (case 8). B: Endoscopic view of one day after 3 L of cola lavage. The size of bezoar was decreased. C: Endoscopic procedure.The remnant bezoar was captured and fragmented into four pieces by basket. D: Endoscopic procedure. The fragmented bezoar was crushed and retrieved by grasping force.

dissolution was observed in four patients (23.5%), whereas 13 cases were partially dissolved with cola, i.e. their size was grossly decreased and their consistency was more softened than that observed before treatment (Figure 1A and B). The clinical characteristics of patients

Table 3 Comparison of clinical characteristics between complete dissolution and partial dissolution groups

	CD group (*n* = 4)	PD group (*n* = 13)	*P* value
Age, range (yr)	51-69	48-78	0.412
Gender (M:F)	0:4	5:8	0.208
Symptom duration, range (d)	14-21	7-90	0.624
Median (d)	20	21	
Type of bezoar			0.006
Phytobezoar	4	2	
Diospyrobezoar	0	11	
Size of bezoar			0.559
Over than 50% of stomach	2	5	
Less than 50% of stomach	2	8	
Endoscopic findings			0.241
GU	3	5	
Gastric outlet stenosis	1	8	
Method of administration of cola			0.441
Lavage	3	6	
Oral drink	1	7	

with complete dissolution (CD) and partial dissolution (PD) are compared and summarized in Table 2. The complete dissolution was observed in 4 out of 6 cases of phytobezoars, but no complete dissolution was noted in diospyrobezoars ($P = 0.006$). Other parameters, including age, gender, size of bezoar, endoscopic findings and the method of cola administration, were not different between the two groups (Table 3). Among the 13 cases with residual bezoars, eleven were completely treated with one endoscopic session and additional drink of three liters of cola. Four patients (case 13, 14, 15, 17) needed two endoscopic sessions to completely disrupt the bezoar.

Thirteen out of 14 (91.6%) patients were completely cured with a combination of cola and endoscopic treatment. The mean endoscopic procedure time was 44.53 ± 18.56 min and the mean number of used accessories was 2.07 ± 1.02. No procedure-

Table 4 Summary of cases for gastric phytobezoar treated with cola

Author	Type of bezoars	No. of cases	Methods of administration of Coca-cola	Duration of Coca-cola
Ladas *et al*[6]	Phytobezoar	5	3 L of Coca-cola lavage	12 h
Kato *et al*[7]	Diospyrobezoars	1	3 L of Coca-cola lavage	12 h
Chung *et al*[8]	Diospyrobezoars	1	Injection of 30 mL of Coca-cola and drinking 4 L of Coca-cola	2 d
Lin *et al*[9]	Diospyrobezoars	1	Injection and irrigation with 1 can of Coca-cola + oral drink of 1 can	
Okamoto *et al*[10]	Diospyrobezoars	1	Drinking 500 mL of Coca-cola per day	7 d
Sechopoulos *et al*[11]	Stump of vegetables	1	2 cans of Coca-cola injection	
Whitson *et al*[12]	Phytobezoar	1	Drinking 5 L of Coca-cola per day	5 d

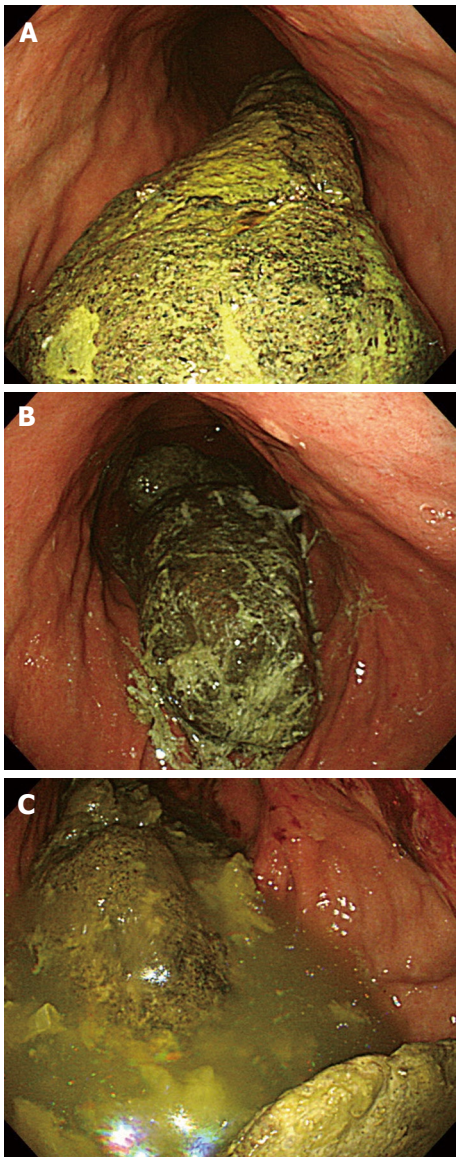

Figure 2 Endoscopic views of huge diospyrobezoar. A: Initial endoscopic view. Endoscopic view of huge dark brownish colored diospyrobezoar in the stomach (Case 17). B: Endoscopic view of one day after 3 L of cola lavage. The size was decreased and some fragmentation was observed. C: Endoscopic view of 4th day. The residual bezoar still remained with fragmentation (total amount of administrated cola was 9 L).

related complications developed, such as hemorrhage, perforation and small bowel obstruction. The mean hospital stay of all patients was 3.52 ± 1.32 d.

In another patient (case 17), the decreased size and softened consistency of the bezoar was observed after an initial lavage with three liters of cola lavage, but the endoscopic breakage failed (Figure 2A and B). So, 100 mL of cola were directly injected into the bezoar using an endoscopic needle and six additional liters of cola were administered orally for two days. On the fourth day, the bezoar still remained in place, with only partial resolution (Figure 2C). Because the patient refused an additional endoscopic treatment with cola, the bezoar was removed by surgery.

DISCUSSION

The treatment modalities for gastric bezoars include endoscopic therapy with fragmentation, medical treatment by enzymatic dissolution and surgery[13]. Various endoscopic methods and instruments for breaking up bezoars have been reported, including lithotripsy with basket[14], endoscopic suction removal with large-channel endoscopy[15], polypectomy snare[16] and biopsy forceps. However, these procedures are time-consuming. Furthermore, procedure-related complications may develop, such as bleeding, overtube-associated complications and intestinal obstruction caused by the fragmented, residual bezoars. In addition, chemical dissolution usually requires a long period of time and complications may develop, such as electrolyte imbalance, gastric ulcer and bleeding. The reported efficacy of chemical dissolution is variable[3,17].

Recently, Ladas *et al*[6] reported a five cases of gastric phytobezoars successfully dissolved by lavage with three liters of cola. Since then, there were six reports written in English and describing the treatment of gastric bezoars with cola. However, reports on cola dissolution have been limited to individual case reports. Methods and results are summarized in Table 4.

In this series, we report the clinical results of 17 gastric phytobezoars treated with cola. To our knowledge, this is largest study ever on this topic. The therapeutic efficacy of a lavage with three liters of cola (or of drinking the same amount), to achieve the complete dissolution of bezoars, was only 23.5%. Compared with previous reports, our success rate is very low. The reason for this low therapeutic efficacy of cola may be due to the fact that most cases of our series were diospyrobezoars (13 out of 17 cases, 76.4%), and in fact we failed to observe the complete dissolution of diospyrobezoars using cola alone.

Diospyrobezoars following ingestion of persimmon are formed by the agglutination of the tannins in the

skin of the fruit. Because of their hard consistency, endoscopic therapy with fragmentation or enzymatic dissolution is challenging and sometimes mechanical fragmentation cannot be accomplished. In a previous report, the efficacy of the combination of endoscopic fragmentation and pharmacotherapy was 80%[18]. There are four reports of cases in whom the complete dissolution of diospyrobezoars was carried out with cola (Table 4). The direct injection of small amounts of cola directly into the phytobezoars is also rapidly effective and safe[6,7,9]. However, in our experience, this technique was not effective for complete dissolving huge disopyrobezoar (case 17). So, in case of diospyrobezoars, complete dissolution might not be achieved with cola use alone.

Another reason for the low therapeutic efficacy may be the relatively short duration of cola administration. There were two successful dissolution cases with daily cola drinking for longer durations (7 d[10] and 3 mo[18]). A prolonged administration may have changed our clinical results, but it may also have induced metabolic disturbances due to the cola's high caloric content. Also, a longer administration time may prolong the *nil per os* time and the length of hospital stay. So, we used the short duration of cola and combined it with the endoscopic fragmentation.

Cola alone could not dissolve completely all gastric phytobezoars. However, in our series, softened-consistency or decreased size was observed in all residual bezoars. So, endoscopic fragmentation and retrieval of the bezoars could be easily performed. Except for four cases, all the procedures were completed in only one session, with relative short procedure time. As the bezoars' consistency was softened, disruption of accessories was prevented. So, these techniques are cost-effective when considering the length of the hospital stay, the number of endoscopic sessions and the used accessories. Also, using additional cola after endoscopic disruption may be helpful preventing small bowel obstruction due to daughter fragments.

Cola's mechanism of dissolution of bezoars is not well understood. The suggested mechanisms are: (1) the mucolytic effect of $NaHCO_3$, (2) the digestion of the bezoar by CO_2 bubbles and (3) the cola's acidity, which is similar to that of gastric acid[6].

In conclusion, the complete dissolution rate using three liters of cola was 23.5%, but no case of diospyrobezoars was completely dissolved. However, pretreatment with cola may be helpful and facilitate endoscopic fragmentation of gastric phytobezoars. A combination therapy of gastric phytobezoars with cola and endoscopic fragmentation is cost-effective and decreases the number of endoscopic sessions and accessories that are used as well as the hospital stay.

COMMENTS

Background

Ladas *et al* have first reported a case series of gastric phytobezoars that were successfully dissolved with cola lavage along with oral maintenance. Since then, there were several reports of gastric bezoar cases successfully treated only with cola.

Innovations and breakthroughs

The efficacy of cola for dissolving bezoars was very low compared with previous reports. There was no case of completely dissolved diospyrobezoar with cola. However, the use of cola for dissolving bezoars was cost-effective.

Applications

This study may suggest that dissolving gastric bezoars with cola alone is not the best treatment modality, especially for diospyrobezoars.

Peer review

The study evaluated the efficacy of cola treatment for gastric phytobezoars. The study is well written and perhaps the largest experience todate.

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BRIEF ARTICLES

TSPAN1 protein expression: A significant prognostic indicator for patients with colorectal adenocarcinoma

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Abstract

AIM: To determine if TSPAN1 overexpression is associated with clinicopathological and prognostic factors in human colorectal adenocarcinoma.

METHODS: Total RNA was extracted in 20 human adenocarcinoma tissues for TSPAN1 mRNA assay by RT-PCR. Eighty-eight specimens of human colorectal adenocarcinoma were surgically removed. TSPAN1 protein levels in cancer tissues were determined by immunohistochemistry using a polyclonal antibody against self-prepared TSPAN1. The correlation between TSPAN1 expression and the clinicopathological factors and the overall survival rate was analyzed by univariate and multivariate assay.

RESULTS: TSPAN1 mRNA was detected in 90.0% (18/20) of cancerous tissues. The light density of TSPAN1 mRNA expression levels was 0.89 ± 0.30 in adenocarcinoma by gel-image system. TSPAN1 protein expression was detected in 78.41% (69/88) and weakly expressed in 40% normal colorectal tissues. There were significant differences between colorectal adenocarcinoma and normal control epithelium ($P < 0.05$). TSPAN1 protein expression in colorectal cancerous tissue was significantly correlated with the histological grade, cell expression PCNA, lymph nodal metastasis and TNM staging of the disease. Patients with TSPAN1 protein overexpression had a significantly shorter survival period than that in patients with TSPAN1 protein negative or weak expression, respectively ($P < 0.05$). Furthermore, by multivariate analysis, TSPAN1 protein expression demonstrated an independent prognostic factor for human colorectal cancers ($P < 0.05$, relative risk 0.755; 95% confidence interval 0.302-1.208).

CONCLUSION: The expression of *TSPAN1* gene is increased in colorectal carcinoma, suggesting that TSPAN1 might serve as an independent prognostic factor for the colorectal adenocarcinoma patients.

Key words: TSPAN1; Colorectal adenocarcinoma; Semi-quantitative RT-PCR immunohistochemistry; Prognosis

Peer reviewer: Jin Gu, Professor, Peking University School of Oncology, Beijing Cancer Hospital, Beijing 100036, China

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INTRODUCTION

The colorectal carcinoma is one of the most common malignant neoplasms, ranking the fourth frequency in men and third in women[1]. Although the prognosis has slightly improved in the past years, colorectal cancer is still the second and third major common cause of

cancer related death in men and women in the United States, respectively[2]. The incidence of colorectal cancer is the fourth in malignant tumor ranking in China, and it is increased dramatically in developing regions[3,4]. The colorectal cancer is thought to result from a combination of environmental factors, diet, lifestyle, chronic inflammation and accumulation of specific genetic alterations. The pathogenesis and development of colorectal cancer involve multi-genes and multi-steps. Ogino *et al*[5] showed the occurrence of colorectal cancer involved in a series of gene mutations, microsatellite instability (MSI) and 18q loss of heterozygosity (LOH). The other molecules studied include MST1 (Mammalian sterile 20-like kinase)[6]. Replication protein (RPA)[7], ELAV-like protein Huk and COX-2[8], α-catenin, β-catenin[9] α-ligatin, β-ligatin, Rho-a[10], *etc*. In fact, an established cascade of events leading to colorectal cancer development and progression is described by Vogelstein. The alteration of expression of these molecules often showed an obvious correlation with pathologic grading and clinical staging in colorectal cancer, which can be used as a biomarker for assessing prognosis. Currently, the assessment of prognosis is mainly based on pathological features of the tumor which is valuable to the triage of patients who will benefit from adjuvant therapy. The clinical pathological staging is the most popular standard prognostic approach for predicting the clinical outcome of colorectal cancer patients[11,12]. The prognosis of colorectal cancer is closely related to the tumor TNM stages. However, patients with similar stages of the disease have various outcomes. Therefore, there is a need to identify useful prognostic molecular markers in guiding treatment decisions and/or in developing more effective treatments. TSPAN1 (GenBank Accession No. AF065388) is a new member of TM4SF[13], which is located at chromosome 1 p34.1. It encodes a 241 amino acid protein. TSPAN1 was reported as a tumor-related gene recently[13-17]. In several studies, TSPAN1 gene over-expression was detected in liver cancer[14], prostate cancer[15], gastric carcinoma[16] and cervix cancer[17]. It has been proposed that TSPAN1 plays a role in cell mitosis and/or cause cell abnormal differentiation. In this study, we examined fresh tumor tissues and histological sections of colorectal adenocarcinoma to determine the expression of TSPAN1 mRNA and protein, and analyzed the relationship between the gene expression and clinicopathological parameters. The result suggests that overexpression of TSPAN1 is correlated to the prognosis of colorectal cancer patients.

MATERIALS AND METHODS

Specimen

A total of 88 patients with colorectal adenocarcinoma, diagnosed and treated from January 1998 to April 2000 were investigated in this study. Of the 88 cases evaluated, 46.6% (41 cases) were rectum cancers, 30.1% (27 cases) were sigmoid colon cancers, 6.8% (6 cases) were descending colon cancers, 2.3% (2 cases) were transverse colon cancers and 13.6 % (12 cases) were ascending colon cancers. The median age at the time of diagnosis was 62.2 years (range, 37-85). There were 50 male patients, 38 female patients. None of them had received chemotherapy or radiotherapy before diagnosis. After surgery, these patients with TMN stage Ⅱ took oral 5-fluorouracil and patients with stage Ⅲ-Ⅳ were subjected to 5-fluorouracil-based systemic chemotherapy. In order to avoid bias, each case was diagnosed by two pathologists.

The clinicopathological data were determined according to the WHO classification and TNM cancer staging[11,12,18]. The average size of the tumor was 4 cm (range from 1.5 to 7.6 cm), 54.5% (48 cases) were cauliflower/polyp type and 45.45% (40 cases) were ulcer/sclerotic type. Adenocarcinomas were graded predominantly on the basis of the extent of glandular appearances, and divided into well (lesions exhibit glandular structures in > 95% of the tumor, grade 1, 15.9% or 14 cases), moderate (lesions have 50%-95% glands, grade 2, 44.31% or 39 cases) and poor differentiation (lesions have 5%-50% glands, grade 3, 39.77% or 35 cases). Tumor limited in submucosa (T1) and muscularis propria (T2) as stage Ⅰ accounted for 32.95% (29 cases), tumor invaded through muscularis propria into subserosa or into non-peritonealized pericolic or perirectal tissues (T3) and tumor directly invades other organs or structures and/or perforates visceral peritoneum (T4) as stage Ⅱ accounted for 29.54% (26 cases), and the tumor with metastasis in 1-3 regional lymph nodes (N1-3) in any T as stage Ⅲ and the tumor with distant metastasis (M) in any T and N as stage Ⅳ, Ⅲ and Ⅳ accounted for 37.5 % (33 cases). Vascular invasion in 26 cases (29.55%) demonstrated that vessel wall was occlusive or infiltrating damaged up to the complete destruction with a surrounding fibroinflammatory reaction[19-21]. Such clinicopathological factors as perineural invasion and desmoplasia reaction were observed and analyzed as well. The proliferation level of cancer cells was evaluated based on the expression of PCNA in tumor parenchymas.

Semiquantitative reverse transcription-polymerase chain (RT-PCR)

Twenty cases of fresh colorectal cancer specimens were stored in -70℃ refrigerator immediately after dissection for semi-quantitative RT-PCR with co-amplification of TSPAN1 gene and an internal control β-actin. Briefly, total RNA from tumor tissues was extracted with TRLZOL reagent and the reverse transcription was performed with Rneasy Kit (Clontech, CA, USA) according to previously published protocols[14]. A 50 μL PCR reaction contains approximately 50 ng of human colorectal cancer ds-cDNA; 40 mmol/L Tricine-KOH, pH9.2; 15 mmol/L KOAc; 3.5 mmol/L Mg $(OAc)_2$; 0.2 μmol/L 5' TSPAN1 primer (5'-CAG-TTC-CCT-CTT-TCA-GAA-CTC-ACT-G-3'); 0.2 μmol/L 3' TSPAN1primer (5'-ATC-CAC-CCA-GAG-GCT-CTG-CTG-ATT-TCA-CCT-3'); 0.1 μmol/L 5' β-actin primer (5'-TTA-CAC-CCT-TTC-TTG-ACA-AAA-CCT-A-3');

0.1 μmol/L 3' β-actin primer (5'-CAA-AAG-CCT-TCA-TAC-ATC-TCA-AGT-3'); 0.2 mmol/L each of dATP, dGTP, dCTP and dTTP; and 1 μL of Advantage™ cDNA Polymerase Mix (50X; contains KlenTaq-1 and Deep Vent polymerases). The PCR cycling was as follows: PCR tubes were preheated at 94℃ for 20 s; then run 30 cycles at 96℃ for 6 s (denature); 60℃ for 20 s for annealing and 72℃ for 1 min for extension, in a DNA thermal cycle 9600 (PE Biosystems, CA, USA). PCR products were applied to electrophoresis on 1% agarose gel analysis; the expected *TSPAN1* gene was a band at 1159 bp. TSPAN1 expression was evaluated by calculating the average ratios of light density using symmetry computerized gel imaging system[14].

Immunohistochemistry

All 88 adenocarcinoma samples were routinely fixed in 40 g/L formaldehyde solution and embedded in paraffin. After slicing into 4 μm thick sections, immunohistochemistry was performed using Dako Elivision TM Plus Two-step System (PV-6000 kit, Zymed, Co., USA.). To detect the TSPAN1 and PCNA expressions in colorectal adenocarcinoma tissues, the sections were dewaxed in xylene and rinsed in alcohol and graded alcohol/water mixtures. Sections were then submitted to antigen retrieval treatment in a pressure cooker. The tissues were boiled in 0.01 mol/L, pH 6.0 citric acid buffer to retrieval antigen for 5 min. They were then treated with 0.3% hydrogen peroxide in absolute methanol to inhibit endogenous peroxidase activity for 15 min at room temperature. After blocking of background staining with diluted normal calf serum, sections were incubated overnight at 4℃ with polyclonal antibodies against TSPAN1 (antibody prepared with the help of American San Francisco gene biotechnology company) and PCNA (PC10, No. 40780708, DAKO, USA), respectively. Subsequent reaction proceeded using a two step assay, immunoreaction was visualized with peroxidase-3,3'-diaminobenzidine (DAB). Finally, sections were lightly counterstained with Mayer's haematoxylin and mounted. The negative controls were set by omitting the primary antibodies. The positive controls were the hepatocellular carcinoma with positive expressions of TSPAN1. In addition, 10 specimens from the marginal normal mucosa of tumor were used as normal controls[16].

Evaluation of immunohistochemical staining

All sections were blindly analyzed by two experienced pathologists under light microscope. Based on the estimated percentages of positive parenchyma cells and/or the immunostaining intensity, which was determined by comparing the immunoreactivity of the positive controls that were included in each experiment, staining results were divided into four categories: (-) tissues specimens: positive parenchyma cell with less than 5% of the cancer tissues and/or weakly stained; (+) tissue specimens: positive parenchyma cell with less than 25% of the cancer tissues and/or weakly stained; (++) tissues specimens: positive parenchyma cell with less than 50% of the cancer tissues and/or moderately stained, and (+++) tissue specimens: positive parenchyma cell with more than 75% of the cancer tissues and/or strongly stained[14,16].

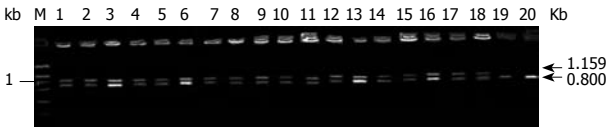

Figure 1 Analysis of TSPAN1 and β-actin mRNA expression in 20 cases of colorectal adenocarcinoma. TSPAN1 and β-actin mRNA expressions were detected in 20 cases of colorectal adenocarcinoma tissues by semi-quantitative RT-PCR. The upper bands were TSPAN1 and the lower bands were β-actin. Lane M: 200 ng of 1 kb size ladder (New England BioLabs); Lanes 1-20: Colorectal adenocarcinoma tissues.

Statistical analysis

Association between *TSPAN1* gene expression and other clinicopathological factors of the tumor were assessed by the Fisher's exact test (two-sided) for categorical variables and χ^2 test were used to compare ordinal variables. The grading-related data was analysed by Spearman test. Overall survival was defined as the period from the date of diagnosis to the date of death. Survival curves were determined according to the Kaplan-Meier method, and compared using Log-rank test statistical differences. Multivariate survival analysis was performed with SPSS version 11.0 Software (Chicago, IL, USA).

RESULTS

RT-PCR detection of TSPAN1 mRNA expression

Total RNA was extracted from 20 cases of colorectal adenocarcinoma tissues. RT-PCR analysis of TSPAN1 mRNA expression was then performed. The positive rate of TSPAN1 mRNA expression was 90% (18/20) in the colorectal adenocarcinoma (Figure 1), and the relative amount of TSPAN1 mRNA levels in cancer tissues was assessed based on the β-actin control. The relative amounts of TSPAN1 mRNA were 0.89 ± 0.30.

Immunochemistry detection of TSPAN1protein expression

TSPAN1 was mainly presented in cytoplasm and located at membrane as well. In the normal control epithelium, 3 cases presented a weakly positive staining of TSPAN1, and only 1 case presented moderately positive expression (Figure 2A). We observed TSPAN1 protein expression in 78.41% (69/88) cases of tumors, in which 17.39 % (12/69) was displayed as strong expressed (+++), 44.93% (31/69) as moderately expressed (++), and 37.68% (26/69) as weakly expressed (+). There were significant differences between colorectal adenocarcinoma and normal control epithelium ($P < 0.05$), (Figures 2B-E).

Correlation with clinicopathological parameters

To investigate the role of TSPAN1 expression in colorectal cancer, we examined the correlation of

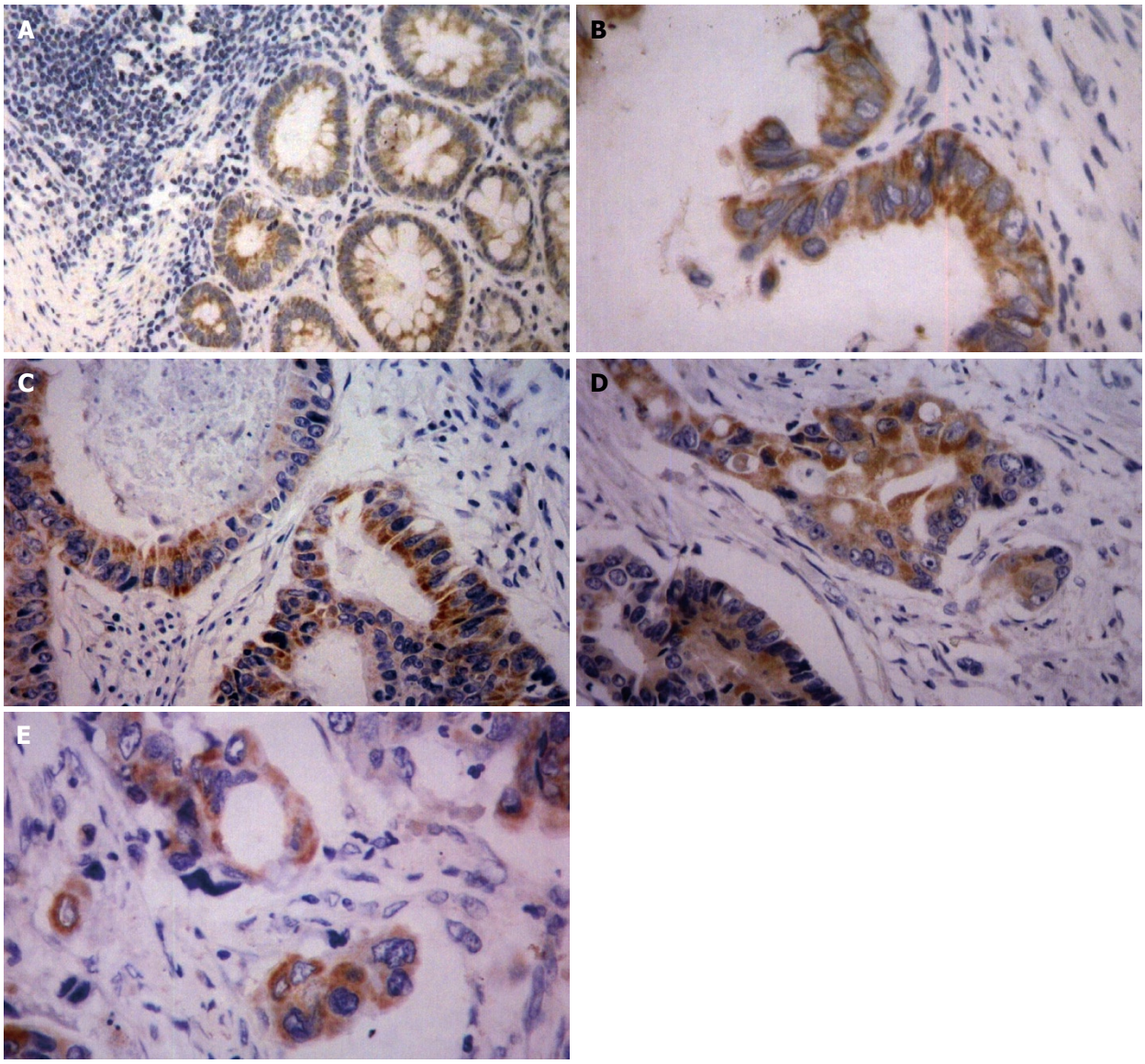

Figure 2 TSPAN1 expression in normal tissues (A), colon cancer tissues (B, C), rectal cancer tissues (D, E). Paraffin section of human colorectal carcinoma tissues was stained with anti-TSPAN1 polyclonal antibody by immunohistochemistry. A: TSPAN1 weakly expressed in the cytoplasm. (× 100). B, C: TSPAN1 was located in the cytoplasm with yellow granulation. (× 200). D, E: Cancer nest showed positive TSPAN1 expression and vascular invasion. (× 200).

TSPAN1 expression with the clinicopathological features (Table 1). We found a positive correlation with histological grade, PCNA expression, nodal metastasis and TNM stages (P = 0.001, 0.015, 0.008 and 0.002, respectively). TNM staging of colorectal cancer is more important for patient's prognosis evaluation. The five-year survival rate of TMN stage 1 is more than 95%, while it is less 10% in patients with TNM stage Ⅲ-Ⅳ. From Table 1, it can be found that the TSPAN1 expression rate and intensity in early TNM stage were lower than in late TNM stage cancer tissues. In addition, TSPAN1 expression was not associated with vascular invasion, perineural invasion and desmoplasia.

Correlation with patients' survival rate

Within a period of 60 mo of the follow-up, 24 cancer-related deaths occurred, 3 of the deaths come from 9 patients with TSPAN1 negative tumors, and 21 from 33 patients in the TSPAN1 positive group. In the entire cohort, the overall survival rate of patients with TSPAN1 negative tumors were significantly higher than that of those with TSPAN1 positive tumors (63.64% *vs* 33.33%; log-rank test: χ^2 = 15.48, P = 0.001). Kaplan-Meier estimated the overall survival rate based on cell TSPAN1 expression in the patients with a follow-up period of 60 mo (Figure 3). To compare with other clinicopathological factors, the effects of histologic grades, node status, PCNA expression, TNM stages, vascular invasion or perineural invasion on the patients' survival were also analysed with univariate log-rank test. As shown in Table 2, the factors of cellular differentiation, node status, PCNA expression, TNM stages had a significant effect on the overall survival rate (P = 0.03, 0.001, 0.0003 and 0.002, respectively). Furthermore, univariate survival analysis was performed to investigate possible prognostic impact of TSPAN1 in

Table 1 Correlation of clinicopathological parameters with TSPAN1 expression

Parameters	Cases	TSPAN1 expression intensity				*P*
		-	+	++	+++	
Gender						
Male	50	9	14	21	6	0.472
Female	38	10	12	10	6	
Tumor size (cm)						
< 4.0	35	10	10	10	5	0.469
> 4.0	53	9	16	21	7	
Type						
Cauliflower/polyp	48	9	14	16	9	0.595
Ulcer/infiltration	40	10	12	15	3	
Location						
Rectum	41	9	11	17	4	0.595
Colon	47	10	15	14	8	
Grade						
Well	14	6	6	2	0	0.001
Moderate	39	9	14	14	2	
Poor	35	4	6	15	10	
PCNA						
+	43	14	14	13	2	0.015
++/+++	45	5	12	18	10	
Lymph node metastasis						
No	55	16	20	14	5	0.008
Yes	33	3	6	17	7	
TNM stage						
Ⅰ	29	11	9	7	2	0.002
Ⅱ	26	5	11	7	3	
Ⅲ-Ⅳ	33	3	6	17	7	
Vascular invasion						
No	62	14	21	20	7	0.424
Yes	26	5	5	11	5	
Perineural invasion						
No	67	16	22	22	7	0.235
Yes	21	3	4	9	5	
Desmoplasia						
No	55	10	16	20	9	0.647
Yes	33	9	10	11	3	

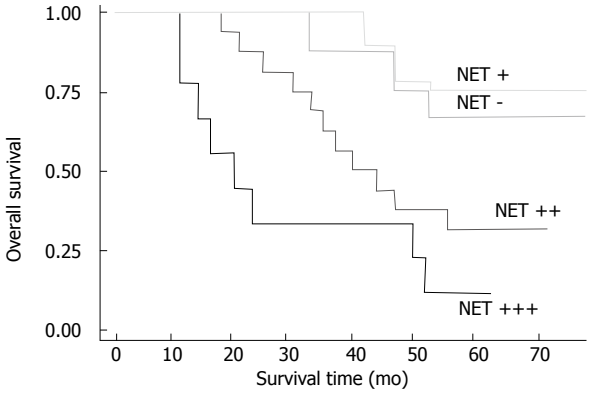

Figure 3 Overall 5-year survival curve of colorectal adenocarcinoma patients with TSPAN1 negative (-) and TSPAN1 positive (+, ++, +++) for the entire cohort (*P* = 0.001) was estimated by Kaplan-Meier test. Survival rate in TSPAN1 expression groups (++, +++) were obviously lower than that of weak expression (+) or negative (-) group, respectively ($P < 0.05$). There was no significant difference of survival rates between TSPAN1 negative group (-) and TSPAN1 weak expression group (+).

colorectal cancer. As shown in Table 2, the expression of TSPAN1 correlated with a worsening of the survival probability, which was statistically significant. This was also confirmed by a multivariate survival analysis including above factors (Table 3). All of these results suggested that TSPAN1 expression in tumors was an independent prognostic factor for colorectal adenocarcinoma patients (relative risk = 0.755; 95% confidence interval: 0.302-1.208 $P = 0.001$).

Table 2 Univariate analysis by Log-rank test

Parameters	5-yr survival rate (%)	Log-rank test	
		χ^2	*P*
TSPAN1 expression			
-	66.67 (6/9)	15.48	0.0015
+	71.4 (5/7)		
++	35.3 (6/17)		
+++	11.1 (1/9)		
Grade			
Well	87.5 (7/8)	6.91	0.0316
Moderate	37.5 (6/16)		
Poor	27.8 (5/18)		
Node status			
No	71.6 (12/17)	15.67	0.0001
Yes	24.0 (6/25)		
PCNA expression			
+	63.1 (12/19)	9.05	0.0026
++-+++	26.1 (6/23)		
TNM stages			
Ⅰ	83.3 (10/12)	16.20	0.0030
Ⅱ	62.5 (5/8)		
Ⅲ-Ⅳ	13.6 (3/22)		
Vascular invasion			
No	46.4 (13/28)	1.39	0.2377
Yes	37.7 (5/14)		
Perineural invasion			
No	44.7 (14/32)	0.77	0.3795
Yes	40.0 (4/10)		
Desmoplasia			
No	33.3 (3/9)	0.02	0.8829
Yes	42.4 (15/33)		

Table 3 Multivariate analysis in Cox proportional hazard model

Variable	Multivariate analysis				*P* value
	HR	SD	Z	95% CI	
TSPAN1 expression	0.755	0.231	3.27	0.302-1.208	0.001
Grade	0.798	0.318	2.51	0.175-1.421	0.012
Node status	1.779	0.509	3.49	0.781-2.778	0.000
PCNA expression	1.325	0.475	2.79	0.394-2.256	0.005
TNM stages	1.159	0.341	3.39	0.490-1.829	0.001
Vescular invasion	0.491	0.423	1.16	0.338-1.320	0.246
Perineural invasion	0.409	0.473	0.87	0.517-1.336	0.386
Desmoplasia	0.061	0.415	0.15	0.752-0.873	0.884

DISCUSSION

Many studies reported that TSPAN1 mRNA and protein were expressed in human normal tissues and carcinomas[13-17]. Serru detected TSPAN1 expression in various cell lines by RT-PCR including cervical cancer, lung cancer, squamous carcinoma, colorectal cancer and breast cancer cells[13]. Wollscheid *et al*[17] detected TSPAN1 mRNA level by RT-PCR and TSPAN1 protein by immunohistochemistry in cervical cancer and found that the gene was expressed in CIN Ⅲ, cervical squamous cell carcinoma and adenocarcinoma,

especially in all undifferentiated cervical carcinoma and adenocarcinoma. They thought *TSPAN1* gene expression correlated to cell proliferation and may be used as a marker for cervical cancer prognosis. However, *TSPAN1* gene expression in human colorectal cancer tissues has not been reported so far. In this study, we for the first time demonstrated that TSPAN1 mRNA and protein were extensively expressed in 90% and 78% human colorectal cancer tissues, respectively. Our results revealed that epithelial cells of the normal colon or rectum displayed a slight expression of TSPAN1 antigen (Figure 2A). There was significant difference between cancer tissues and normal control. The results are consistent with most other reported data[12-14] and suggest that the TSPAN1 expression is a specific marker for malignant transformation.

In colorectal cancer, the presence of many tumor-associated antigens and their relationship with clinical pathological parameters have been described[22-23]. PCNA, a major marker for cell proliferation, is highly expressed in most tumors[24]. In this study, the finding of a significant positive correlation between TSPAN1 and PCNA expression provided further evidence to support a potential role of TSPAN1 in tumor proliferation process (Table 1). The colorectal cancer development may hence relate to the accumulation of TSPAN1 protein in tumor cells. Similarly, our previous study found that TSPAN1 expression correlated with tumor proliferation maker Ki67 expression in human gastric carcinomas[16].

Currently, the TNM stage represents the main tool for identifying prognostic differences among patients with colorectal cancer. The reported 5-year survival rate is 95% for stage Ⅰ patients, 67% for stages Ⅱ, and 9.4% for stage Ⅲ and Ⅳ patients[25]. In our prospective 5-year follow-up study, the overall survival rate was 83.3% for stage Ⅰ patients, 62.5% for stage Ⅱ patients, and 13.6% for stage Ⅲ and stage Ⅳ patients (Table 2). Similarly, we showed that there was a significant correlation between the overall survival rate and the disease stages. Our study revealed that there was a statistically significant association between TSPAN1 expression and the various stages of colorectal cancer, in which TSPAN1 positive staining was seen in 63.64% patients with shorter survival time (Table 3). The univariate and multivariate analyses suggested that TSPAN1 status, PCNA expression, tumor stages and nodal status were strong predictors for the final clinical outcome (Table 3). Likewise, another study in our lab also showed that TSPAN1 expression was significantly correlated with the metastasis and poor prognosis of gastric carcinoma[16]. Increasing TSPAN1 protein expression was found associated with more advanced stages of cervical carcinoma[17]. All these findings suggest that TSPAN1 over-expression status might yield unfavorable prognosis for some types of cancers. Identifying those patients with high-risk colorectal cancers by TSPAN1 expression detection would be of great benefit for improving the treatment strategies. By the way, other reports displayed that vascular invasion and perineural invasion were correlated with a poor prognosis[19-21], but in this study we found no direct effect on tumor prognosis.

Colorectal carcinoma is one of the most common cancers in western world and in China, however its molecular mechanism is still unclear. To understand the specific regulation of gene expressions between colorectal cancer and non-cancer tissues and know the genes or proteins characteristics will delineate the molecular changes and obtain useful diagnostic marker. We have demonstrated that TSPAN1 was expressed in majority of human colorectal carcinomas in the current study. TSPAN1 expression, measured by immunohistochemistry in the tumor tissues, may be a candidate gene for diagnosis and prognosis of colorectal carcinoma. The overexpression of TSPAN1 in cytoplasm is associated with higher tumor grade, metastasis, proliferation, and more advanced stages and poor prognosis in colorectal adenocarcinoma patients, suggesting a tumor-related gene role of TSPAN1 in human colorectal cancer development.

ACKNOWLEDGMENTS

The authors thank Mrs Lu for her help in collecting survival data.

COMMENTS

Background

The colorectal cancer results from a combination of environmental factors, diet, lifestyle, chronic inflammation and accumulation of specific genetic alterations. TSPAN1 (GenBank Accession No. AF065388) is a new member of TM4SF located at chromosome 1 p34.1. It encodes a 241 amino acid protein. TSPAN1 was reported as a tumor-related gene recently.

Research frontiers

TSPAN1 gene over-expression was detected in liver cancer, prostate cancer, gastric carcinoma and cervix cancer. It has been proposed that TSPAN1 plays a role in cell mitosis and/or cause cell abnormal differentiation.

Innovations and breakthroughs

In this study the authors examined fresh tumor tissues and histological sections of colorectal adenocarcinoma to determine the expression of TSPAN1 mRNA and protein, and analyzed the relationship between the gene expression and clinicopathological parameters, and found that overexpression of TSPAN1 is correlated to prognosis of colorectal cancer patients.

Applications

Testing TSPAN1 expression in tissues would be a useful tool to evaluate the prognosis of patients with colorectal cancer.

Peer review

The authors examined the expression of Net-1 in colorectal tissues, a novel gene whose function has yet to be understood so far. This study is of some clinical significance.

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CASE REPORT

Jejunoileal bypass: A surgery of the past and a review of its complications

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Abstract

Jejunoileal bypass (JIB), popular in the 1960s and 1970s, had remarkable success in achieving weight loss by creating a surgical short bowel syndrome. Our patient had an unusual case of liver disease and provided no history of prior bariatric surgery. Later, it was recognized that he had a JIB in the 1970s, which was also responsible for the gamut of his illnesses. Patients with JIB are often not recognized, as they died of complications, or underwent reversal of their surgery or a liver-kidney transplant. Early identification with prompt reversal, and the recognition and treatment of the life-threatening consequences play a critical role in the management of such patients.

Key words: Jejunoileal bypass; Bariatric surgery; Weight loss; Obesity; Morbid obesity

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INTRODUCTION

Bariatric surgery is one of the few proven methods that cause durable weight loss. Failure of conservative means of producing permanent weight reduction in patients with morbid obesity, led to the introduction of operative approaches, such as jejunoileal bypass (JIB), which became popular in the late 1960s and early 1970s[1].

At that time, JIB was the most effective surgical intervention for achieving and maintaining weight loss. Typically, 35 centimeters of proximal jejunum was anastamosed, end-to-side or end-to-end, to the terminal 10 centimeters of ileum[2] (Figure 1). It was presumed that patients undergoing this procedure would experience continued hyperphagia, but would accomplish weight loss due to malabsorption[3]. As a result of JIB, patients whose preoperative weight was over 157 kg lost a mean of 58 kg at the end of 1 year[2].

However, JIB surgery has long been abandoned as a method of weight reduction surgery because of serious short and long-term complications. The number of patients who currently retain a jejunoileal bypass is small, as most patients have died or undergone reversal of their operation or conversion to a different bariatric procedure[3]. Recognition of previous JIB and understanding of its metabolic consequences are essential in the proper management of these patients.

CASE REPORT

A 64-year-old male was admitted on a regular basis for tense ascites (requiring serial large volume paracentesis) attributed to underlying advanced liver disease of unclear etiology. It was presumed to be the result of steatohepatitis from nonalcoholic fatty liver disease and/or chronic hepatic congestion due to decreased cardiac function.

The patient had a prior history of morbid obesity (191 kg, BMI 62) and cholecystectomy. His physical examination was remarkable for jaundice, abdominal ascites, spider angioma in the upper chest, gynecomastia and splenomegaly.

He had numerous other medical problems including multiple kidney stones with three previous lithotripsy interventions, progressive kidney disease requiring hemodialysis, a 30-year history of intermittent loose

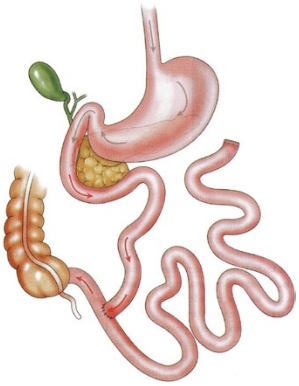

Figure 1 Jejunoileal bypass. A surgical short bowel syndrome created by bypassing more than 90% of the functioning small intestine.

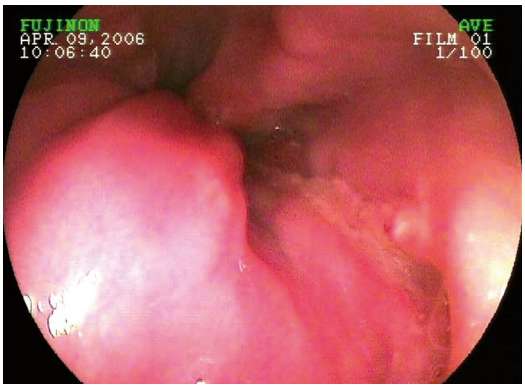

Figure 3 Upper endoscopy showing grade I esophageal varices, as a result of portal hypertension.

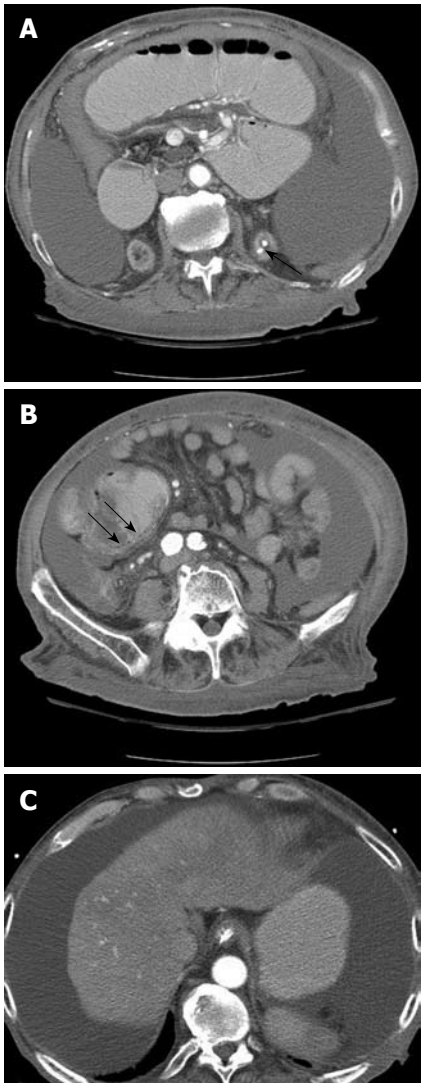

Figure 2 CT scan of the abdomen. showing stones in the left kidney (A, arrow), pneumatosis intestinalis (B, arrow) and shrunken nodular liver with abdominal ascites (C).

Table 1 Laboratory investigations

BUN/Creatinine (normal = 8-26/0.9-1.3)	34/8.2
Albumin (normal = 3.5-5.0)	1.9
Prothrombin time/INR (normal = 11.9-14.3/< 1.0)	25.3/2.4
ALT/AST (normal = 15-41/14-63)	45/41
Platelet count (normal = 140-400)	129
Serum albumin ascites gradient	2.3
Hepatitis profile (A, B, C)	Negative

BUN: Blood urea nitrogen; INR: International normalized ratio; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase.

stools, arthritis, fatigue, paresthesias, progressive loss of night vision, joint pains, slurred speech, incoordination and weakness.

On further questioning, the patient and family revealed that he had "weight loss surgery" in the 1970s at another facility. However, an upper endoscopy showed an intact pylorus, which raised suspicion of possible previous JIB surgery. His archived medical records were then obtained and it revealed that the patient indeed had a jejunoileal bypass performed in 1974.

Initial laboratory evaluation is noted in Table 1. Further evaluation revealed deficiency of all fat-soluble vitamins. The markers of other causes of chronic liver disease (viral hepatitis B and C, antinuclear antibody, anti-smooth muscle antibody, anti-mitochondrial antibody, ceruloplasmin, iron and ferritin) were negative. Stool studies showed findings consistent with steatorrhea. Computed tomography imaging of his abdomen revealed nephrolithiasis, pneumatosis intestinalis, a shrunken and nodular liver with abdominal ascites, and osteopenia (Figure 2). Upper endoscopy, as in Figure 3, revealed esophageal varices, portal hypertensive gastropathy and no evidence of prior gastric bypass.

He underwent a transjugular liver biopsy that showed portal fibrosis (stage 2, grade 1) with specimen fragmentation. A repeat transjugular liver biopsy with hepatic hemodynamic measurements was performed, with a hepatic portal venous gradient at 7 mmHg (normal, 1-4 mmHg), the wedge pressure was 23 (normal, 4-13 mmHg) and the hepatic vein pressure was 16 mmHg (normal, 2-10 mmHg) - findings consistent with portal hypertension.

The patient declined reversal of JIB and did not wish evaluation for liver-kidney transplantation. He is currently being managed symptomatically by hospice care.

The complications of JIB are summarized in Table 2 above[4].

Table 2 Complications of jejunoileal bypass

Problem	Mechanism	Manifestations in this patient
Steatohepatitis Possible cirrhosis	Amino acid deficiency	Advanced liver disease with portal hypertension
Renal oxalosis	Excess oxalate absorption; Oxalate not bound by calcium	Multiple kidney stones and three previous lithotripsy interventions; Progressive kidney disease due to suspected oxalate nephropathy requiring lifelong hemodialysis
Fat soluble vitamin deficiency	Malabsorption; Steatorrhea	Serum levels: Vitamin A = 17 (360-200 mg/L) Vitamin D ⩽ 10 (22-67 pg/mL) Vitamin E = 3 (5.5-17.0 mg/L) Vitamin K ⩽ 0.03 (0.1-.2 ng/mL)
Gallstones	Bile acid loss; Mobilization of cholesterol	Previous cholecystectomy for symptomatic cholelithiasis
Enteritis	Bacterial overgrowth	30 years of diarrhea and steatorrhea Pneumatosis intestinalis
Arthritis	Bacterial toxin; Autoimmune	Bilateral knee and shoulder pain
Fatigue syndrome	Vitamin deficiency; Multifactorial	Marked fatigue, bed-ridden status
Bypass encephalopathy	Possible deficiency; Possible D-lactic acid deficiency	Slurred speech, incoordination and weakness
Bypass dermatitis	Possible antigen-antibody complex (enteric bacteria)	Cutaneous urticarial rash

CONCLUSION

Although jejunoileal bypass was effective and reliable, it was associated with severe complications such as renal failure (37%), diarrhea (29%) and consequent electrolyte imbalances, calcium oxalate nephrolithiasis (29%), liver disease (10%), fat-soluble vitamin deficiencies, malnutrition and death[5]. The most severe early complication of JIB was acute liver failure (7%)[5]. Our patient exhibited nearly all of the known metabolic complications described in the literature (Table 2).

Of the various types of bariatric procedures, JIB is particularly devastating because of its dramatic complications, and is no longer used for the management of morbid obesity. A number of studies[6-8] highlighted the high complication rates even after a decade and sometimes lifelong, difficulty maintaining satisfactory follow-up and the need for frequent revision surgery making it an unacceptable procedure performed remotely on any major scale.

Today, this procedure is a formidable diagnostic challenge because it was a surgery of the olden days (1960s and 1970s), old records may not be available and upper endoscopy may be essentially normal. Most patients have either died or had conversion to a different bariatric procedure. The importance lies in early identification based on clinical history, with prompt reversal and the recognition and treatment of the life-threatening metabolic consequences.

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CASE REPORT

Embolization of an unusual metastatic site of hepatocellular carcinoma in the humerus

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Abstract

Hepatocellular carcinoma (HCC) is the fifth most common cancer in the world. This case documents an unusual metastatic presentation of HCC in the humerus. Preoperative palliative arterial embolization of the tumor was performed to arrest severe tumor bleeding caused by the biopsy. Embolization turned out to be useful also in limiting/preventing potential uncontrolled bleeding during subsequent amputation.

Key words: Hepatocellular carcinoma; Humerus; Upper arm; Metastasis; Embolization

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer in the world[1]. The most frequent sites of extrahepatic metastases of HCC are the lungs, lymph nodes, bones, and adrenal glands[2], whereas the extremities, and especially the humerus, are very rare metastatic sites. Here we report an interesting case of humerus metastasis of HCC with severe hemorrhage after biopsy.

CASE REPORT

A 74-year-old man with a background of alcoholism and smoking initially presented with pain on the right side of the chest. Clinical examination revealed mild hepatomegaly and pallor. An abdominal ultrasound showed the presence of a solid mass in the right lobe of the liver, measuring 5.2 cm × 6.5 cm, with heterogeneous echotexture (Figure 1A). Computed tomography (CT) imaging of the abdomen confirmed the presence of a lesion in segment Ⅷ of the liver, with central areas of necrosis (Figure 1B). An additional, smaller focal lesion was found also in segment Ⅶ of the liver (Figure 1C). Both lesions showed typical imaging characteristics of an HCC. Notably, a thrombosis of the portal vein was also present (Figure 1D), which presented a contraindication for chemoembolization of the HCC. The patient was discharged.

After 4 mo, the patient was referred again with progressive painful swelling of the left upper arm and superficial ulceration. An X-ray showed a destructive lesion of the left humerus, associated with a bulging soft tissue component (dimensions 5.5 cm × 4 cm). The diaphysis of the humerus was completely destroyed for a length of 4 cm (Figure 2A and B). Magnetic resonance imaging (MRI) clearly demonstrated a large osteolytic lesion (coronal dimensions 6.2 cm × 5 cm) in the left humerus space, with complete destruction of the bone (Figure 2C and D). The tumor reached the surface of the skin. A surgical biopsy was performed to sample tissue from the ulcerated mass. However, massive bleeding developed from the biopsy site immediately after excision. Because the hemorrhage could not be stopped by electrocauterization, it was decided to perform an arterial embolization on the assumption that possible HCC metastases would typically be hypervascularized. A 5 French cobra catheter was advanced from the femoral artery into the left axillary artery and a selective

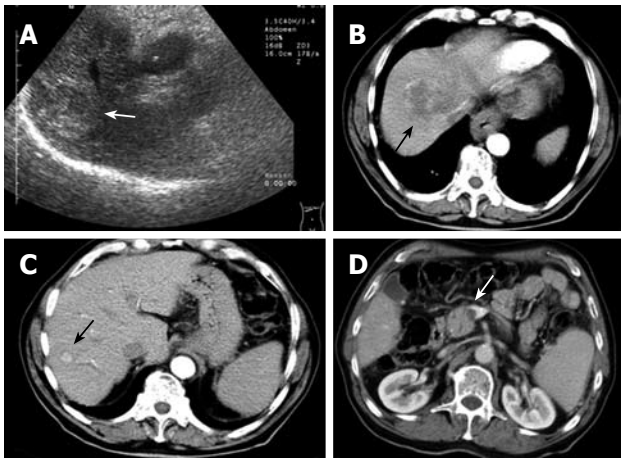

Figure 1 Liver sonography (A) and CT imaging of hepatocellular carcinoma (HCC) (B-D). A: Round but inhomogeneous tumor with hyper- and hypo-echoic appearance in the sonography (diameter 6 cm, arrow); B: Axial multidetector CT contrast image at the arterial phase demonstrated a tumor lesion in the right upper lobe (arrow). Dimension 7 cm × 6.5 cm × 60 cm with hyperdense periphery and hypodense centre (latter areas of necrosis); C: Additional 1.4 cm × 1 cm × 1 cm hyperintense lesion in segment Ⅶ of the liver (arrow); D: Partial thrombosis of the portal vein (arrow).

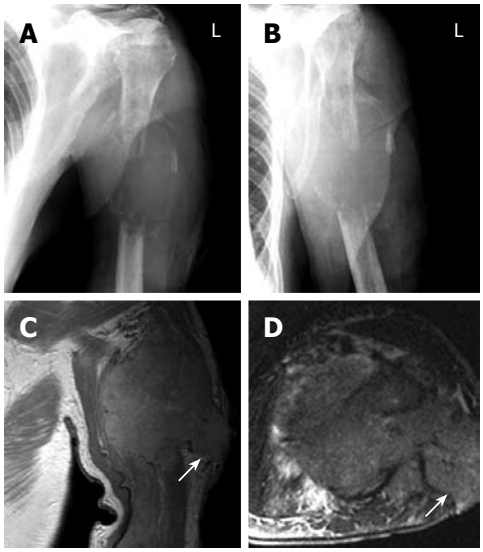

Figure 2 X-Ray (A, B) and MR imaging (C, D) of HCC metastasis at the humerus. A and B: Destructive lesion in the left humerus associated with a bulging soft tissue component, 5.5 cm × 4 cm in dimension; C: Coronal noncontrast PD-weighted (TR/TE, 1920/12) image demonstrating a soft tissue tumor (coronal dimension 6.2 cm × 5 cm) with complete destruction of the humerus, the tumor reached the surface with ulceration (arrow); D: Axial noncontrast TIRM sequences (TR/TE, 7400/92) show the edematous tumor in the centre of the extremity and the lateral tumor branch to the surface (arrow). In this region, a biopsy was taken which was followed by massive hemorrhage.

arteriogram was obtained. Injection of contrast medium showed a hypervascular, destructive tumor of the humerus (Figure 3A). Extravasation of the contrast medium indicated the presence of hemorrhage (Figure 3B). Afterwards, multiple accessible feeding branches were reached with superselective catheterization using a tracker catheter, and embolized with Bead Block (size 300-500 μm, Terumo Europe, Leuven, Belgium). Follow-up angiographies during embolization showed stasis of flow within the tumor. At

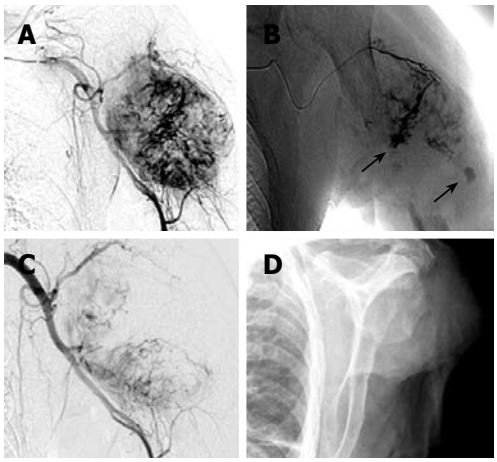

Figure 3 Catheter angiography of the tumor before (A, B) and after embolization (C), X-Ray after final treatment with amputation of the left upper extremity (D). A: Catheter angiography of the axillary artery reveal a round hypervascular tumor; B: Selective angiography of a tumor feeding vessel as side of application of Bead Block (size 300-500 μm, Terumo Europe, Leuven, Belgium) for embolization; C: After embolization of main parts of the tumor, at this status no areas of bleeding detectable but small parts of the tumor still perfused; D: Final treatment with amputation of the left upper extremity.

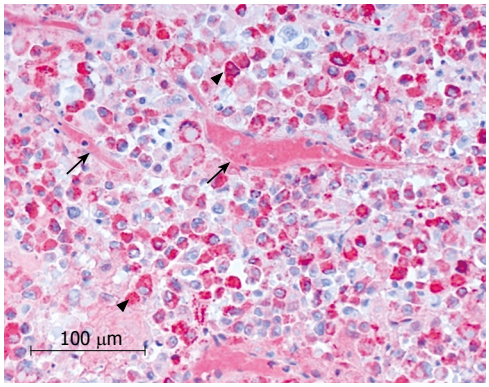

Figure 4 Neoplastic cells of HCC metastasis were diffusely stained by hepatocellular antigen (for instances, see arrowheads), destroyed bone areas within the tumor are visible (arrows).

the end, partial tumor embolization was achieved. The medial parts of the tumor remained perfused, since not all the small-sized branches of this region could be accessed or localized (Figure 3C). However, bleeding was successfully stopped. There were no postprocedural complications. On the following day, amputation of the left arm was deemed necessary since an alternative treatment, e.g. osteosynthetic stabilization of the destroyed humerus, was not achievable (Figure 3D). Thus, *a posteriori* the embolization proved useful also as a measure to prevent uncontrolled hemorrhages during amputation.

The histopathological evaluation of the biopsy confirmed the diagnosis of metastasis from the liver HCC (Figure 4).

DISCUSSION

This report describes an unusual case of massive metastasis of a HCC carcinoma to the upper arm, with partial

destruction of the humerus. To the best of our knowledge, no such cases have been reported in the literature to date. Also, while bone metastases of HCC usually manifest as multiple lesions, no other bone metastases were detected in this case.

The prognosis of HCC patients with extrahepatic metastasis is generally poor[3]. It is estimated that 30%-78% of HCC show metastases at autopsy[4]. HCC spreads mainly *via* the hematogenous route, causing intra- and extra-hepatic metastases that are generally hypervascular and, if located in the bone, osteolytic. Hypervascularity should be taken into account before biopsy excisions since the procedure can cause an uncontrolled hemorrhage, as reported here and by Chen *et al*[5] who described a life-threatening hemorrhage from a sternal metastasis of HCC. In the present case, the marked size of the tumor in the upper arm did not allow us to distinguish whether the metastasis had primarily localized in the muscle or in the bone. However, intramuscular HCC metastases are extremely rare[6,7], possibly because of the contractile action of muscle, its local pH environment, and/or the accumulation of lactic acid. Also, skeletal muscle produces tumor suppressors which may contribute to the rarity of metastases in the muscles[8]. A study with 194 autopsies of malignant tumors showed that macro- or micro-metastases of skeletal muscle were present only in 34 cases (17.5%)[9]. Metastases of HCC are clearly more frequent in the bone but, unlike the present humerus case, their most common sites are the vertebrae, the pelvis, and the ribs. In turn, bone metastases of HCC are commonly characterized by expansive soft tissue masses with bone destruction, as in the present case.

To date, only a few reports are available concerning arterial embolization of bleeding HCC metastases. This procedure can support treatment to reduce the tumor size[10] or to reduce the severity of the symptoms (e.g. pain or bleeding). Wallace *et al*[11] reported that patients with bone metastases who underwent embolization had a reduction of pain for 4-9 mo. In our case, the goal of embolization was to control the bleeding following biopsy excision. A posteriori, a benefit was also the containment/prevention of potential bleeding during amputation. Amputation became necessary because of the advanced tumor size and the degree of bone destruction.

In conclusion, this case documents an unusual metastatic presentation of HCC in the humerus. Preoperative palliative arterial embolization of the tumor was performed to arrest severe tumor bleeding caused by the biopsy. Embolization turned out to be useful also in limiting/preventing potential uncontrolled bleeding during subsequent amputation.

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CASE REPORT

Repair of a mal-repaired biliary injury: A case report

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Abstract

Iatrogenic bile-duct injury post-laparoscopic cholecystectomy remains a major serious complication with unpredictable long-term results. We present a patient who underwent laparoscopic cholecystectomy for gallstones, in which the biliary injury was recognized intraoperatively. The surgical procedure was converted to an open one. The first surgeon repaired the injury over a T-tube without recognizing the anatomy and type of the biliary lesion, which led to an unusual biliary mal-repair. Immediately postoperatively, the abdominal drain brought a large amount of bile. A T-tube cholangiogram was performed. Despite the contrast medium leaking through the abdominal drain, the mal-repair was unrecognized. The patient was referred to our hospital for biliary leak. Ultrasound and cholangiography was repeated, which showed an unanatomical repair (right to left hepatic duct anastomosis over the T-tube), with evidence of contrast medium coming out through the abdominal drain. Eventually the patient was subjected to a definitive surgical treatment. The biliary continuity was re-established by a Roux-en-Y hepatico-jejunostomy, over transanastomotic external biliary stents. The patient is now doing well 4 years after the second surgical procedure. In reviewing the literature, we found a similar type of injury but we did not find a similar surgical mal-repair. We propose an algorithm for the treatment of early and late biliary injuries.

INTRODUCTION

Laparoscopic cholecystectomy (LC) has emerged as a gold standard of cholecystectomy and is the commonest laparoscopic surgical procedure performed by many surgeons worldwide. Unclear anatomy of the biliary tract and acute cholecystitis are associated with an elevated risk of bile duct injuries. These are serious surgical complications and are sometimes unrecognized during the procedure. A clear interpretation of the biliary anatomy as well as a good surgical experience are prerequisites for a definitive surgical repair. Primary surgical repair and further misinterpretation of the biliary anatomy with consequent mal-repair of the biliary tract injury are very unusual conditions. We present here a case of biliary tract injury that was recognized during LC but that was submitted to a mal-repair surgical procedure causing difficult problems of correct interpretation and management for the definitive surgical repair.

CASE REPORT

A 45-year-old woman was admitted to the referring hospital with symptomatic gallbladder stones for elective LC. She had not undergone any previous operations. Cholecystectomy was reported to be difficult by the operating surgeon. He reported that she had acute cholecystitis, and the biliary anatomy was not clear. During the operation he recognized that he caused a biliary injury, and for this reason he decided to convert

the intervention to open surgery. He completed the cholecystectomy, and performed a repair of the biliary injury over a T-tube, according to his interpretation of the injury. He did not perform a cholangiogram before the repair, but the post-repair intraoperative cholangiogram was interpreted as a good repair. An infra-hepatic drain was inserted. During the first postoperative day, the abdominal drain brought out 500 mL of bile and the T-tube drained 100 mL of a similar fluid. On the following days, the abdominal drain brought 800-1000 mL of bile daily and the T-tube brought 40-60 mL daily. During the next 2 wk, the output through the drains did not decrease, and the treating surgeon asked to transfer the patient to our hospital, with a diagnosis of biliary fistula. The referring surgeon had an average experience in laparoscopic surgery. Past medical history revealed a non-insulin-dependent diabetes mellitus on oral medication.

In our hospital, the patient appeared pale, mildly dehydrated, not jaundiced and with normal vital signs. She was in the average weight range. The abdominal examination revealed a right sub-costal scar, two scars from the trocars, and two drains (an abdominal drain and the other was a T-tube) that both contained bile. She did not look septic.

The laboratory results showed: hemoglobin 10 g/dL; white blood cell count 11 300/mm^3; K^+ 3.2 meq/L; Na^+ 132 meq/L. Liver enzymes and bilirubin were within the normal range.

Abdominal US showed normal liver size and echogenicity, without intra-hepatic biliary dilatation, a small sub-hepatic collection, and sub-hepatic multiple clips. The distal part of the biliary tree was not identified.

A T-tube cholangiogram (Figure 1) was done in our hospital, which showed a grade Ⅳ Bismuth injury, with the T-tube limbs within the left and right hepatic ducts. The biliary contrast medium appeared immediately through the abdominal drain, and the distal part of the biliary tree was not shown. These findings (mal-placed T-tube and fistula) suggested a plan for the definitive biliary surgical repair. We did not perform magnetic resonance cholangiography (MRC), because the patient already had a T-tube, and also she had a sub-hepatic collection.

Informed consent was obtained from the patient and her family. She was given a prophylactic third-generation cephalosporin, which was continued for 24 h postoperatively. She was shifted to insulin therapy from the day she was admitted to our hospital, which was continued until she returned to her normal diet, on postoperative day 7. The intraoperative finding was a Bismuth grade Ⅳ injury that was repaired by right and left hepatic duct end-to-end anastomosis over a T-tube, performed by the referring surgeon. During exploration, we found an absence of the biliary confluence and the common hepatic duct, which may have indicated a misinterpretation by the first surgeon of the common hepatic duct as the cystic duct, therefore, he excised this region altogether with the gallbladder. Multiple clips on the distal biliary tree (common bile duct) were found.

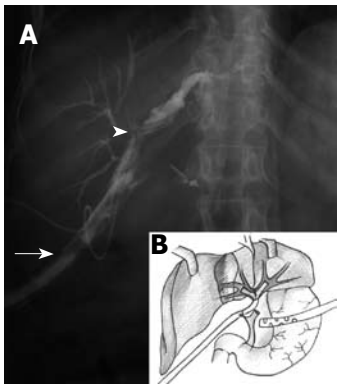

Figure 1 T-tube cholangiography. A: Postero-anterior T-tube cholangiogram showing biliary tract mal-repair (arrowhead indicates the T-tube, arrow indicates the tube drain); B: Schematic diagram of the cholangiogram.

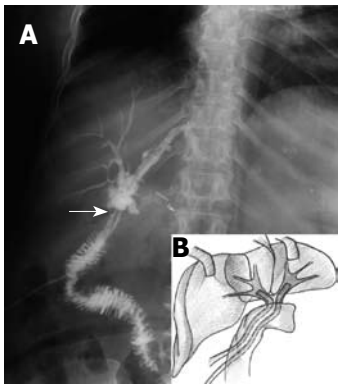

Figure 2 T-tube cholangiography. A: Postero-anterior view of the T-tube cholangiogram, which was done in our hospital, after the definitive surgical repair of the biliary tract injury (arrow: two intra-bilio-jejunal stents); B: Schematic diagram of the cholangiogram.

We also noted a bile leak from the posterior wall of the anastomosis that was performed previously over the T-tube. The T-tube was removed and a Roux-en-Y hepatico-jejunostomy over two trans-anastomotic stents was then performed (Figure 2). The patient had a good postoperative recovery without complications. The trans-stents' cholangiogram performed on postoperative day 10 showed patent anastomosis without leakage. The stents were closed and the patient was discharged. The tubes were removed after 2 mo. The patient was followed up by clinical examination, liver enzymes and abdominal ultrasound (US), every 3 mo for the first year, every 6 mo for the second year, and then every year thereafter. In her final follow-up visit in August 2008, she was in good condition, 4 years after our surgical repair. She did not have any wound complications, neither in the early nor in the late postoperative period.

DISCUSSION

Misinterpretation of biliary anatomy was the main cause of biliary mal-repair. The hard task was to understand this mal-repair carried out by the referring surgeon, and then to perform a definitive biliary repair. The only possible explanation was that the referring surgeon misinterpreted the left hepatic duct as the common bile duct. This was very hard to recognize from the first radiological study, performed in the referring hospital, in which the contrast medium spilled out into the abdominal drain, and it was interpreted incorrectly as the common bile duct. As a result of the poor quality of this radiological study, we performed another T-tube cholangiogram and discussed the case with our

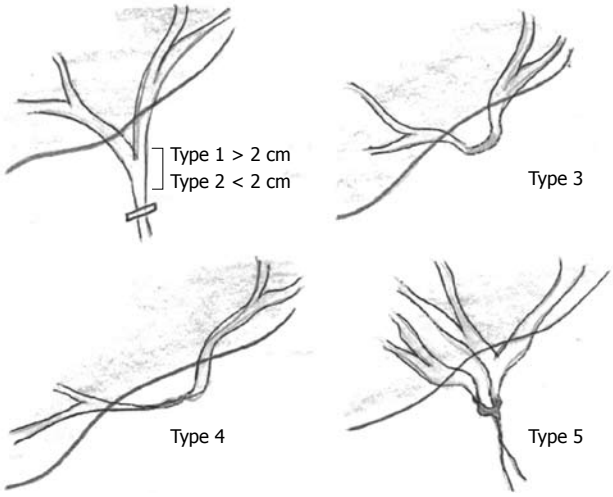

Figure 3 Bismuth classification of biliary injuries.

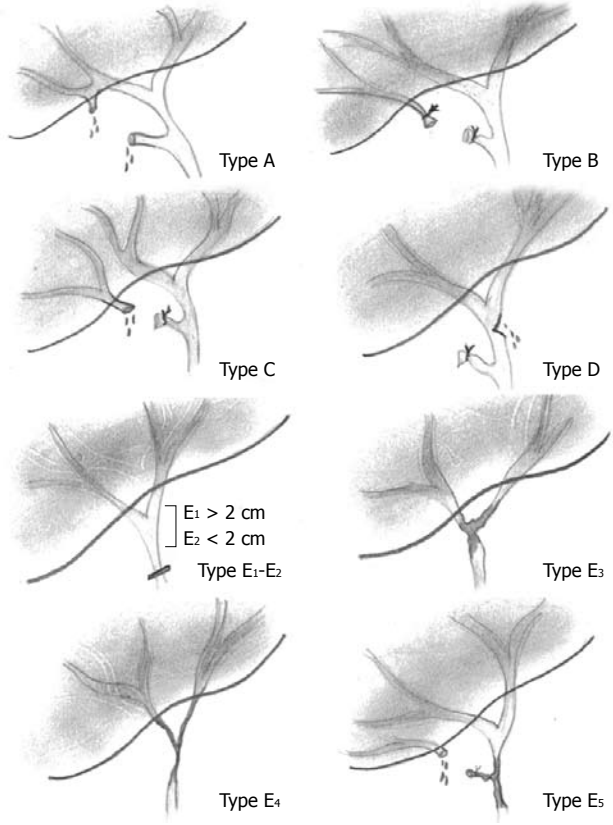

Figure 4 Strasberg classification of biliary injuries.

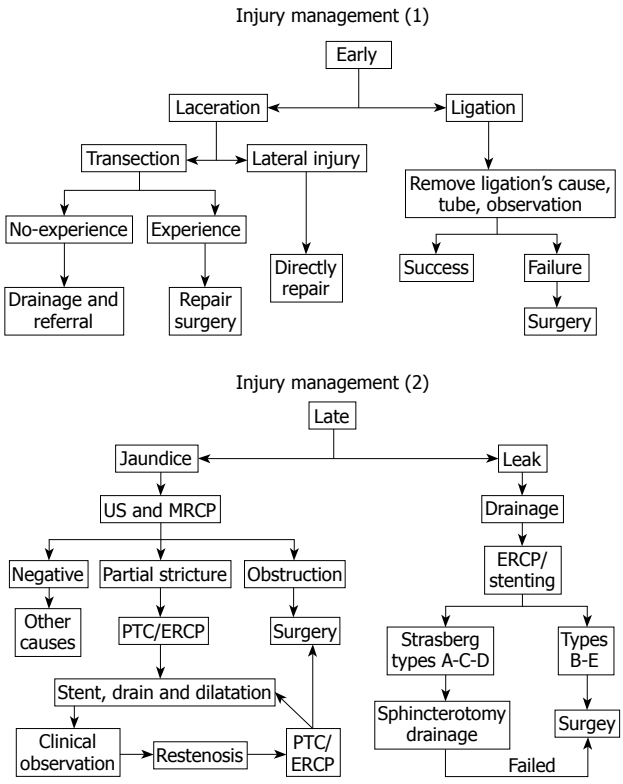

Figure 5 Our suggested flow chart for the management of early and late presentations of biliary injuries. PTC: Percutaneous transhepatic cholangiography; ERCP: Endoscopic retrograde cholangiopancreatography.

radiologist, who confirmed our suspicion of the mal-repair. Although this kind of injury is well known (Bismuth type Ⅳ), we could not find a similar case of mal-repair in the English literature.

The incidence of biliary tract injury ranges from 0.2% to 0.8% worldwide, and even an experienced surgeon can cause such injury[1-5]. There is more than one classification for biliary injury, but the most widely used for surgical purposes are the Bismuth and Strasberg classifications (the later includes the former).

Our patient presented with grade Ⅳ Bismuth or Strasberg E Ⅳ biliary injury, which required surgical treatment. Figures 3 and 4 show drafts for both Bismuth and Strasberg classifications[4,6,7].

The referring surgeon tried to repair the injury immediately during the first operation. The identification of injury during surgery is reported to be between 15% and 50% of cases. Although primary repair is possible, and can be done laparoscopically, it should only be performed by an experienced surgeon. If this is not the case, drainage and referral to an experienced surgeon is the rule[2,3,8-10]. Poor identification of the anatomical features of the hepatic triangle represents the commonest cause of injury[3]. Bismuth noted that the level of the stricture found during repair was one level higher than the level of injury identified during the first operation[7]. During the re-operation for failed repair, the level of stricture has been reported to be even higher[11].

In our case, we re-established the biliary continuity by a Roux-en-Y hepatico-jejunostomy, after trimming the previous biliary anastomosis, over two stents inserted into the right and left hepatic ducts. A postoperative tube cholangiogram is shown in Figure 4. Different methods of repair have been reported by different surgical teams[5,7,12]. Percutaneous and endoscopic retrograde cholangiopancreatography (ERCP) interventions can both be used in the management of biliary injuries, but under different conditions than ours[13]. We recommend, in cases that present with suspected stricture, to start with MRCP as the procedure of choice to identify the anatomy and the type of injury. In our specific case, the patient already had a drain in her biliary system, and she also had a biliary leak, therefore, it was appropriate to

use these drains for delineating the biliary anatomy by a tube cholangiogram.

We propose an algorithm (Figure 5) for the treatment of early and late biliary complications. We consider complications that appeared during the first month postoperatively as early (usually leaks), and those presenting after one month as late complications (usually strictures).

Bile duct injuries are serious surgical complications. A major study led by Cameron showed post-repair mortality of 1.7%, with a similar percentage of patients dying before repair, as a result of sepsis. The study of Cameron also showed a complication rate after repair of 42.9%, despite an early referral to a specialist center[12]. Long-term outcome after repair showed good results in > 90% of cases[8].

In conclusion, biliary tract injuries are sometimes difficult to recognize, even for experienced surgeons. In the absence of an experienced surgeon, it is mandatory to limit the surgical manipulation to simple drainage, and to refer the patient to a more specialized center, in order to give the best chance for definitive treatment.

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CASE REPORT

Mucosal Schwann cell "Hamartoma": A new entity?

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Author contributions: Annibali D and Gimbo G provided the patient; Pasquini P, Baiocchini A, and Del Nonno F performed the histological and immunohistochemical analyses; Pace F participated in acquisition of data; Falasca L and Del Nonno F carried out the design of the study; Falasca L, Del Nonno F and Baiocchini A drafted the manuscript.
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Abstract

Schwannoma is a well-described, benign nerve sheath tumor of the soft tissue, but is rare in the gastrointestinal tract. Gastrointestinal schwannomas are often incidentally discovered as small polypoid intraluminal lesions. In this report, we describe the clinicopathologic and immunohistochemical features of a distinctive neural mucosal polyp composed of a diffuse cellular proliferation of uniform bland spindled cells in the lamina propria that entraps the colonic crypts. Immunohistochemical analysis revealed strong and diffuse positivity for the S-100 protein. To avoid confusion of these solitary colorectal polyps containing pure spindled Schwann cell proliferation in the lamina propria with neural lesions that have significant association with inherited syndromes, it is better to use the designation "mucosal Schwann hamartoma".

Key words: Nerve sheath tumors; Gastrointestinal Schwannoma; Hamartoma

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INTRODUCTION

Schwannoma is a common soft tissue tumor, but it appears to be rare among spindle cell mesenchymal tumors of the gastrointestinal tract[1-4]. Colorectal schwannomas are uncommon, and are incidentally discovered as small polypoid intraluminal lesions, often with mucosal ulceration, during colonoscopy screening. Gastrointestinal schwannomas have characteristic histological features that are different from their soft tissue counterparts, such as the presence of a reactive lymphoid peripheral cuff, the absence of encapsulation and degenerative changes[5-7]. The tumors are mainly situated in the muscularis propria of the digestive wall. Rectal bleeding, colonic obstruction, and abdominal pain are the most common presenting symptoms. The separation of GI stromal tumors (GISTs) from gastrointestinal schwannoma is clinically important because the former group have a high risk of malignant behaviour[8-11], while the second are benign. Recently the designation "mucosal Schwann cell hamartoma" has been proposed for lesions containing diffuse pure Schwann cell proliferation in the lamina propria, which entrap adjacent crypts, to avoid confusion with the neural lesions that are associated with inherited syndromes such as von Recklinghausen's neurofibromatosis[12]. In this study, we report a case of a colorectal polyp comprising diffuse Schwann cell proliferation in the lamina propria that belongs to the entity proposed.

CASE REPORT

Clinical presentation

A 60-year-old woman with no personal history of malignancy underwent a colonoscopy during the workup of occult blood in the stool. She had no family history of colon cancer and no history of familiar adenomatous polyposis, multiple endocrine neoplasia type Ⅱb, neurofibromatosis type Ⅰ, or Cowden syndrome.

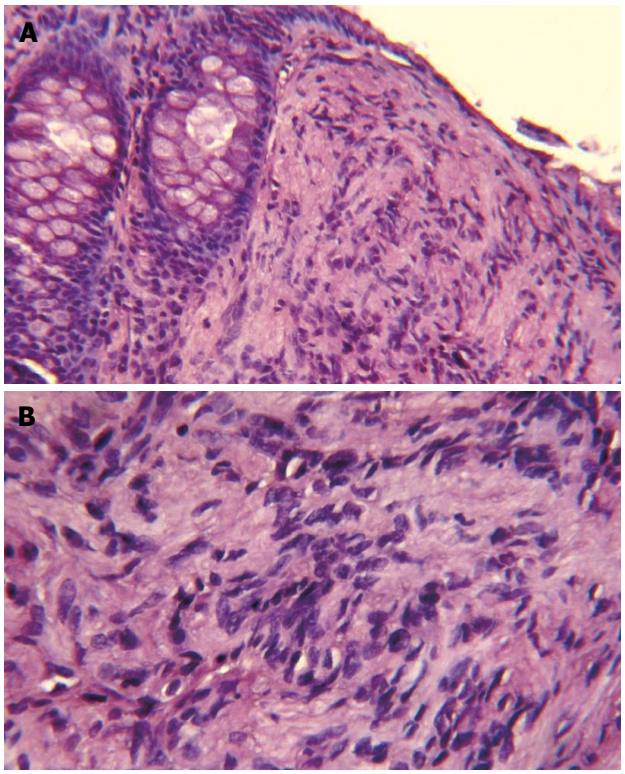

Figure 1 Histological features of the lesion. Low- (A) and high (B)-power magnification of haematoxylin and eosin stained tissue sections of colorectal mucosa. A diffuse, Schwann cell proliferation in the lamina propria, which entraps colonic crypts is visible. Cytologically, the lesions are composed of uniform bland spindle cells with elongated nuclei, dense eosinophilic cytoplasm, and minimal intervening stroma with vague Verocay bodies.

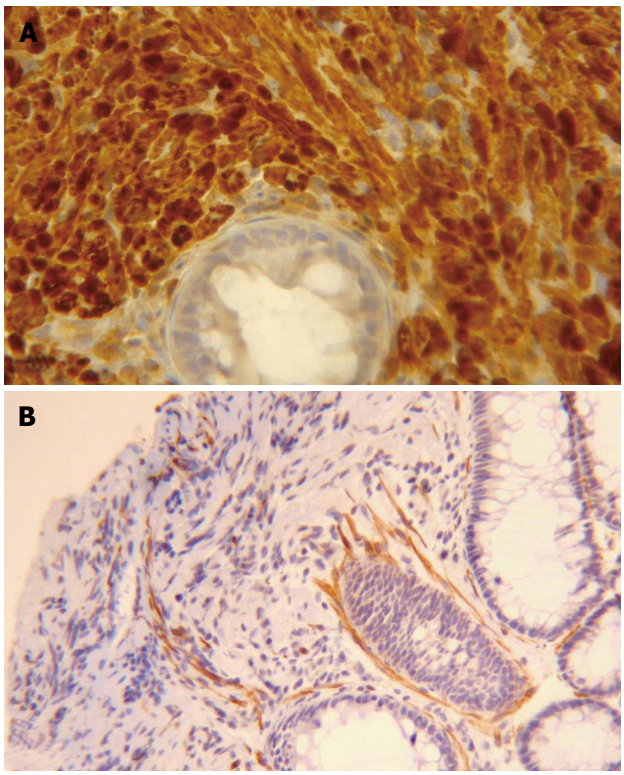

Figure 2 Immunohistochemical staining. The lesion consists of a pure population of Schwann cells, as shown by the diffuse immunoreactivity for the S-100 protein (A). Only scattered myoepithelial cells and vascular structures were highlighted by the immunostaining for α-smooth muscle actin (B).

Endoscopic and microscopic findings

Endoscopic examination showed a small sessile polyp of 0.5 cm in diameter without mucosal ulceration in the rectosigmoid colon. A biopsy was obtained. Hematoxylin and Eosin stained histologic sections showed a diffuse cellular proliferation of uniform spindle cells with elongated, tapering nuclei, and indistinct cell borders, arranged in whorls and vague Verocay bodies, entrapping adjacent crypts (Figure 1). The epicenter of the lesion was located in the lamina propria without involvement of the muscularis mucosae. No nuclear atypia, pleomorphia, or mitoses were seen. The immunohistochemical analysis demonstrated that all the cells were extensively and strongly positive for the S-100 protein (Figure 2A). Cells were negative for CD117 (KIT), α-smooth muscle actin (1A4) (Figure 2B), and CD34 (QBEND/10). Scattered mild chronic inflammation with the rare appearance of mast cells was present in the background.

DISCUSSION

Schwannoma (or neurilemoma) is an encapsulated nerve sheath tumour, common in the soft tissue. In the gastrointestinal tract schwannomas are rare and non-encapsulated, although well circumscribed[3]. They may appear as a small intramucosal nodular lesion, polypoid lesions, or poorly demarcated transmural proliferations[13]. According to the reports of Hou *et al*[7] and Daimaru *et al*[1] schwannomas were more frequently found in the stomach than in the colon or rectum. Miettinen and colleagues[3] and Lewin and colleagues[14], have described and characterized the largest series of colorectal schwannomas. Spindle cells variants have been found to be the most frequent, but epithelioid and plexiform schwannomas have also been described.

Herein we described a case of colorectal polyp in a female patient discovered during colonoscopy for the workup of occult blood in the stool. Histological examination of the polyp revealed a poorly circumscribed proliferation of uniform bland spindle cells, arranged in whorls and with a poorly formed area suggestive of Verocay bodies. The epicenter of the lesion was in the lamina propria, without involvement of the muscularis mucosae. Cellular spindle cell proliferation was interspersed between colonic crypts. No peripheral lymphoid aggregates, nuclear atypia nor mitosis were seen. The proliferated cells were strongly S-100 positive, and CD117, α-smooth muscle actin and CD34 negative, corresponding to the Schwann cell phenotype.

Mucosal neural polyps with similar features to our case have been recently described by Gibson *et al*[12], and we agree with author's proposal of the new and interim designation of "mucosal Schwann cell hamartoma" for this lesion, to avoid confusion with the gastrointestinal neural lesions that have significant associations with inherited syndromes[15-17]. Our case should not be considered as an intramucosal schwannoma, although qualitatively composed exclusively of Schwann cells, because

it presents peculiar histological features: the lack of circumscription, the absence of a peripheral lymphoid cuff[5], and crypt entrapment. Solitary ganglioneuromas and perineuriomas might predominantly involve the mucosa but the population of ganglion cells and the positivity for epithelial membrane antigen (EMA) are distinctive features. In contrast to neurofibromas, the polyp evaluated in our study was cytologically uniform, and, based on diffuse immunoreactivity for S-100 protein, seems to be composed essentially of a pure population of Schwann cells. Moreover, CD34 and neurofilament are useful stains for the differential diagnosis, because neurofibromas typically demonstrate a significant subpopulation of CD34-positive stromal cells and scattered axons.

Leiomyomas might also be encountered arising in association with the muscularis mucosae of the colon. They express desmin, calponin, and caldesmon but lack S-100 protein[14].

It is important in the GI tract to recognize GI stromal tumors (GISTs), which might pursue a malignant course. GISTs might have neural differentiation but they are typically reactive with c-kit/CD117 antibodies, and originate from or differentiate into the interstitial cells of Cajal with activating mutations in the KIT24-27 and PDGFRa genes[7-10].

In conclusion, the lesion we have encountered should be categorized as a mucosal Schwann cell hamartoma. Accurate histological differential diagnosis of this kind of lesion has clinical relevance, not only for immediate patient management, but also because it might provide the first clue to the existence of inherited tumor syndromes (searching for ganglion cells), which will have broader implications for the patient's family and potentially important consequences for genetic counselling.

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CASE REPORT

Fibrosing cholestatic hepatitis following cytotoxic chemotherapy for small-cell lung cancer

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Abstract

Fibrosing cholestatic hepatitis (FCH) is a variant of viral hepatitis reported in hepatitis B virus or hepatitis C virus infected liver, renal or bone transplantation recipients and in leukemia and lymphoma patients after conventional cytotoxic chemotherapy. FCH constitutes a well-described form of fulminant hepatitis having extensive fibrosis and severe cholestasis as its most characteristic pathological findings. Here, we report a case of a 49-year-old patient diagnosed with small-cell lung cancer who developed this condition following conventional chemotherapy-induced immunosuppression. This is the first reported case in the literature of FCH after conventional chemotherapy for a solid tumor. In addition to a detailed report of the case, a physiopathological examination of this potentially life-threatening condition and its treatment options are discussed.

Key words: Fibrosing cholestatic hepatitis; Immunosuppression; Chemotherapy; Lung cancer; Hepatitis B virus; Lamivudine

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INTRODUCTION

Hepatitis B virus (HBV) is a well-known pathogen which can cause fulminant hepatitis in some patients undergoing cytotoxic chemotherapy. Patients who have completely recovered from acute hepatitis can harbor a latent HBV infection for decades[1].

Fibrosing cholestatic hepatitis (FCH) is a recognized unique variant of viral hepatitis which was originally reported in 1991 by Davies *et al*[2] in HBV-infected recipients of liver allografts. Other authors have also reported FCH cases in HCV-infected recipients after liver transplantation[3] and in renal[4], cardiac[5] or bone marrow transplanted patients[6] as well as in patients with acquired immunodeficiency syndrome[7]. FCH has additionally been reported secondary to hepatitis C allograft reinfection[8].

However, to our knowledge only 3 cases of FCH have been reported after conventional cytotoxic chemotherapy. Nonetheless, all of them were diagnosed with non-solid tumors (acute myelogenous leukemia[9], acute lymphoblastic leukemia[10] and low grade non-Hodgkin's lymphoma[11]). FCH is associated with extremely high mortality[12].

We report a case of a patient diagnosed with small-cell lung cancer who developed FCH under chemotherapy-induced immunosuppression.

CASE REPORT

This is a case of a 49-year-old male with a 38 pack-year smoking history and a past medical history of hepatitis B. He had not received previous blood transfusions. At the time of admission, the patient presented with pain in the right hemithorax. Initial investigations included thoracic X-ray, thoracic computed tomography (CT) and positron emission tomography (PET)-CT imaging demonstrating right pleural effusion, right upper lobe

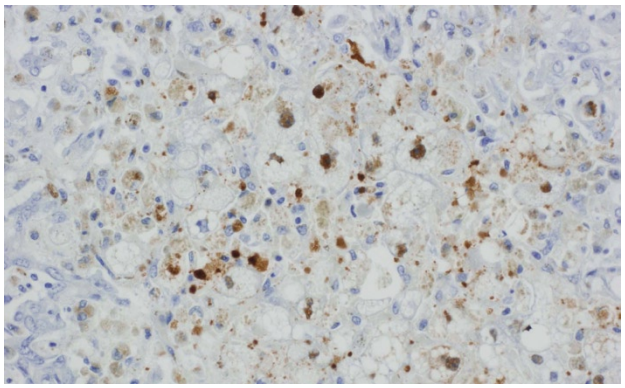

Figure 1 Liver necropsy stained with antibody against hepatitis B core antigen. FCH is also characterized by massive expression of hepatitis B core antigen (nuclear and cytoplasmic).

atelectasia, mediastinal adenopathies and liver and bone metastases. After a bronchoscopy-guided biopsy, he was diagnosed with extensive-disease small-cell lung cancer. The patient accepted chemotherapy and a combination regimen of cyclophosphamide 400 mg/m^2 (days 1-3), adriamycin 40 mg/m^2 (day 1), cisplatin 100 mg/m^2 (day 2), vincristine 2 mg (day 3) and etoposide 100 mg/m^2 (days 1-3) was administered. After the first cycle, the patient presented with grade Ⅳ febrile neutropenia in spite of pegfilgrastim prophylaxis. For this reason, a 20% dose reduction was applied on the second cycle. Doses were escalated to the original levels in the third cycle, during which the patient presented with elevation of glutamic oxalacetic transaminase (GOT) (232 UI/mL) and glutamic pyruvic transaminase (GPT) (639 UI/mL).

Following the third cycle, a PET-CT scan showed a radiological complete response. In contrast, the patient showed persistent elevation of transaminases (GOT: 198 UI/mL; GPT: 400 UI/mL).

After administration of the fourth cycle, he developed grade 4 thrombocytopenia and neutropenia. A complete serological study was performed demonstrating hepatitis B reactivation (HBsAg, HBeAb and HBcAb IgG positive). Despite treatment with the nucleoside analogue entecavir and methylprednisolone, the hepatitis progressed towards acute hepatic failure (GOT: 1879 UI/mL; GPT: 2663 UI/mL; total bilirubin: 42 mg/dL; direct bilirubin: 37 mg/dL; indirect bilirubin: 5 mg/dL; prothrombin time: 10%). A liver biopsy was not performed, due to thrombocytopenia and clotting time elevation.

The patient was transferred to the Intensive Care Unit and received plasma exchange, vasoactive drugs and wide spectrum antibiotics (piperacillin-tazobactam). In spite of these therapies, he died a month after the last chemotherapy cycle from acute liver failure.

The autopsy showed residual tumor in the mediastinum and peribronchial nodes, a hepatic reaction and fibrosing cholestatic hepatitis. The microscopic study revealed marked hepatocyte degeneration, whereas the immunohistochemical analysis demonstrated hepatocytes diffusely stained for HBsAg following both intracytoplasmic and cytoplasmic membranous patterns and nuclear and intracytoplasmic HBcAg staining patterns (Figure 1). Serum levels of HBV DNA polymerase were 17 857 100 UI/mL.

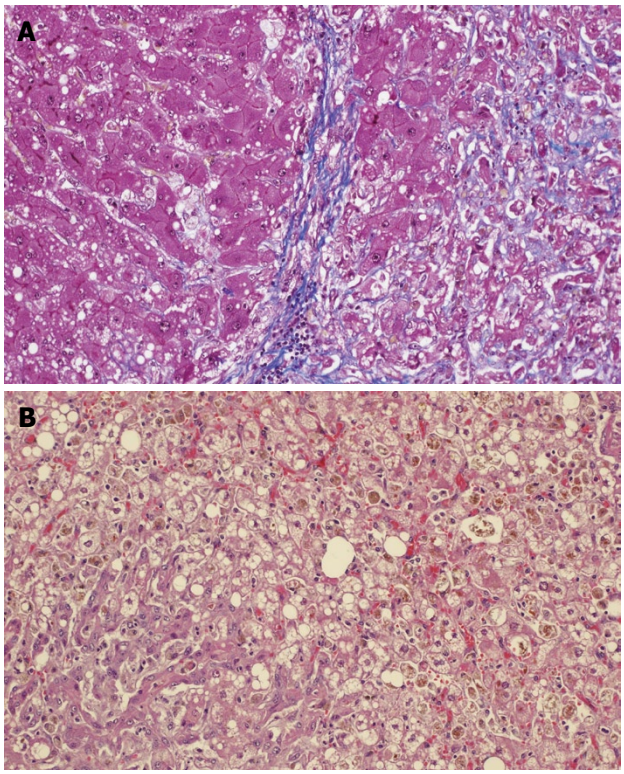

Figure 2 Liver necropsy. A: Stained with Mason trichromic; B: Stained with eosin-hematoxylin. FCH is characterized by marked hepatocellular swelling, lobular disarray and cholestasis, with only mild or no portal or lobular inflammation, combined with acute cholangiolitis and fibrosis surrounding the cholangioles.

DISCUSSION

To our knowledge, this is the first reported case of FCH after conventional cytotoxic chemotherapy treatment in a patient diagnosed with a solid tumor. There have only been 3 case reports published of FCH patients who had received conventional chemotherapy for hematological malignancies. FCH is a subtype of viral hepatitis in HVB-infected patients which is associated with extremely high mortality.

Degeneration of hepatocytes with minimal infiltration of inflammatory cells (which is clearly distinguishable from other fulminant hepatitis), extensive fibrosis and severe cholestasis are the most characteristic pathological findings. Liver parenchymatous changes include hepatocyte swelling and cholestasis with marked ductular reaction[13] (Figure 2A and B).

Although the ultimate physiopathological mechanism in this condition remains elusive[14], the extremely high levels of viral replication, the massive HBcAg and HBsAg expression in the liver and the non-significant inflammatory component suggest a direct HBV cytopathologic effect. The accumulation of viral antigens in the endoplasmic reticulum damages vital cell functions leading to cell death[13]. Although specific treatment protocols are lacking, a few case reports

have described a clinical benefit after administration of ganciclovir/foscarnet antiviral therapy[15] in these patients. Additionally, other reports describe the efficacy of lamivudine, a nucleoside analog reverse transcriptase inhibitor, in the treatment of chronic hepatitis[12] and in prophylaxis of chronic hepatitis and FCH[16]. Moreover, lamivudine has been recommended by some authors for the prophylaxis of HBV hepatitis in carrier subjects undergoing cytotoxic chemotherapy for lymphoid malignancies[17], although no data from clinical trials are available.

This case report suggests that viral analysis might be indicated in patients presenting with solid tumors before initiating intensive chemotherapy regimens. In addition, prophylactic lamivudine should be considered in HVB chronic infection carrier patients (HBsAg positive and/or HBcAb IgG positive) in this setting.

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CASE REPORT

Successful use of adalimumab for treating fistulizing Crohn's disease with pyoderma gangrenosum: Two birds with one stone

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Abstract

Crohn's disease (CD) is a chronic relapsing and remitting autoinflammatory disorder of the gastrointestinal tract that has many intestinal and extraintestinal complications. The purpose of treatment is long-term remission, reduction of complications, and improvement of patients' quality of life. In many cases, this can be quite challenging and it is necessary to have a well thought out management strategy. We present the case of a 38-year-old woman with fistulizing CD that manifested as diffuse abdominal pain and bloody diarrhea accompanied by arthralgia. In addition, there were ulcerative lesions surrounded by cutaneous inflammation and erythema on her extremities, indicative of pyoderma gangrenosum. The patient was treated with high doses of parenteral methylprednisolone without any improvement and was started on adalimumab. A positive response to adalimumab therapy was observed: after 2 mo of therapy, the ulcerative skin lesion healed completely and the enterogastric fistula was closed after 5 mo adalimumab treatment. Adalimumab might be a suitable initial as well as maintenance therapy in patients with complicated CD.

Key words: Adalimumab; Crohn's disease; Pyoderma gangrenosum

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INTRODUCTION

Crohn's disease (CD) is characterized by fissuring ulcers and segmental transmural inflammation of the gastrointestinal tract. The ileum is frequently involved in chronic inflammatory diseases; however, these can occur in any part of the digestive tract, from mouth to anus. Fistulas are the major and most common complications of the disease. The cumulative risk of any kind of fistula is 33% after 10 years and 50% after 20 years from the first appearance of the disease, as exemplified by a population-based study[1]. Although CD predominantly affects the gastrointestinal system, it is also associated with several extraintestinal manifestations. The most common extraintestinal disorders associated with inflammatory bowel disease (IBD) include dermatologic, ophthalmologic, musculoskeletal and hepatobiliary diseases; however, practically every organ system could be involved. These extraintestinal disorders can significantly contribute to morbidity and consequently impair the overall life quality of the patient considerably more than bowel-related symptoms.

Treatment is very complex and includes antibiotics and various immunomodulators. Surgery may be needed for therapy-refractory cases. The increasing number of advanced biological treatments for IBD offers new possibilities for the management of IBD associated with extraintestinal manifestations[2].

We report a case of successful adalimumab usage for CD complicated by enterocutaneous fistula, which also constituted an effective alternative treatment for pyoderma gangrenosum.

CASE REPORT

A 38-year-old woman presented with a 20-year history of CD, numerous complications and frequent relapses. Previously she had undergone several corrective gastrointestinal surgeries for perirectal fistulas. She carried a stoma since 1997 following left hemicolectomy and Hartmann's procedure. She continued to receive maintenance 5-aminosalycilate (mesalamine) and budesonide therapy. Corticosteroid therapy and combined antibiotic/antimycotic treatment were used intermittently for increased disease activity resulting in moderate clinical response. The patient was unable to tolerate azathioprine.

In February 2004, she developed erythema nodosum on her extremities, which was resolved by corticosteroid-antibiotic treatment. The patient was referred to our department for further examination and management in November 2004, when she developed asymmetric oligoarthritis that responded well to treatment with a corticosteroid and maintenance methotrexate, resulting in remission for 6 mo. In 2005, the patient was treated with intravenous pulse cyclophosphamide for moderate to severe disease activity as a rescue therapy.

The patient remained in remission for 6 mo. In January 2007, she was admitted to our hospital again because of increased weakness, mild ulcerative skin lesions, and abdominal pain with bloody diarrhea. Additionally, she had arthralgia but no fever. Her blood tests showed a white cell count of 8.97×10^9 cells/L, a platelet count of 289×10^9 cells/L, a C reactive protein level of 68.57 mg/L, a hematocrit of 36 and an increased erythrocyte sedimentation rate (64 mm/h). Immunologic evaluation did not show B-cell/immunoglobulin disorders or antibody positivity. Upon physical examination, she had some small, red papules on her extremities with ulcerations on their surface that were characterized by pyoderma gangrenosum (Figure 1). In addition, we also found diffuse abdominal tenderness and an abdominal mass located in the periumbilical region. Her urine and chest X-rays were normal. Abdominal computed tomography (CT) showed a moderately enlarged spleen as well as thickened and inflamed bowel walls that are characteristic of CD (Figure 2). The inflamed bowels were surrounded by fat stranding, and CT also showed multiple enterocolic fistulas. Upper endoscopic examination revealed an enterogastric fistula from the corpus of the stomach to the stoma. Surgical correction was not an option because the patient did not agree to surgery. The patient received infliximab (Remicade; Schering-Plough) therapy as well as maintenance methotrexate therapy but there was no significant improvement in her symptoms and in her test results.

Following a failed course of infliximab (because of lack of response), adalimumab (Humira; Abbott Laboratories) was proposed as an alternative treatment. Adalimumab was administered subcutaneously at a dose of 80 mg/wk for the first 2 wk followed by 40 mg/wk afterwards. In addition, the patient was treated for a limited period with high doses of parenteral methylprednisolone and a combined antibiotic treatment. After 2 mo of therapy, the skin ulcers were completely healed. Subjective clinical improvement could be seen after 3 mo of therapy. The patient did not experience any adverse effects. After 5 mo of therapy contrast radiography of the bowel passage showed significant improvement. A 3-4 cm long, narrow and blind fistula originating from the stomach could be seen but it was not connected to the bowel. Nevertheless, the duodenum and jejunum were moderately inflamed and the terminal ileum was intact without inflammatory signs.

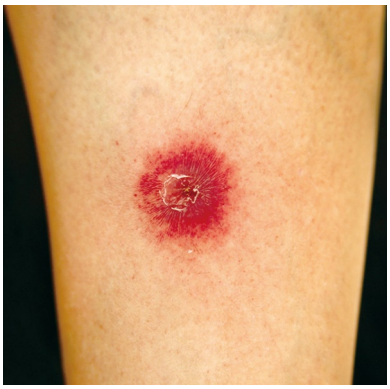

Figure 1 Small, red papules on the extremities with ulcerations.

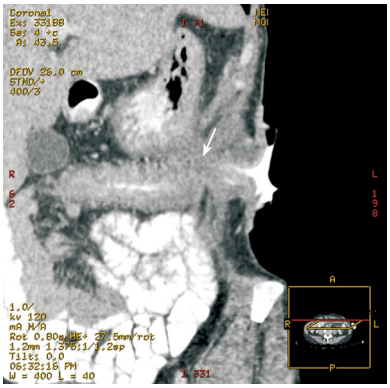

Figure 2 Enterogastric fistula from the corpus of the stomach to stoma on abdominal computed tomography (white arrow).

DISCUSSION

Up until now the use of adalimumab for fistulizing CD with extraintestinal manifestations has been reported in only a few cases. We reported a case of a woman with CD complicated by fistulas and pyoderma gangrenosum. The patient was successfully treated with adalimumab.

Extraintestinal manifestations of IBD can be diagnosed before, simultaneously with, or after the diagnosis of IBD is made. These symptoms occur in 21%-36% of IBD cases. It is important to distinguish complications of IBD from secondary diseases as they demand different and specialized therapies. The recognition of symptoms can be difficult since these extraintestinal symptoms can also be the primary manifestations of IBD[3,4].

The connection between different extraintestinal symptoms and IBD is unclear. The shared and unique epitopes in the human colon, eye, joint and biliary epithelium may suggest an immune mediated process.

Pyoderma gangrenosum is one of these inflamma-

tory cutaneous manifestations and it is independent of disease activity. This ulcerative cutaneous condition can either be associated with systemic inflammatory diseases (in at least 50% of cases) or it can occur alone. The diagnosis is made by excluding other causes of cutaneous ulcerations that are similar in appearance, including infection, malignancy, vasculitis, collagen vascular diseases, diabetes, and trauma. Treatment is relatively difficult. Currently, systemic immunosuppressants, often prednisone, are the mainstay of therapy. Long-term therapy with these agents is often required and can expose patients to possible adverse effects.

The treatment of IBD with extraintestinal manifestation has advanced in parallel to our increasing understanding of its pathomechanism[5-9]. One of the most well recognized proinflammatory mediators involved in the pathogenesis of IBD is tumor necrosis factor α (TNF-α). It is known that high levels of TNF-α have been associated with the development of intestinal inflammation in CD. The current evidence suggests that TNF-α blocking agents such as infliximab, adalimumab, and certolizumab pegol are effective maintenance therapy in CD. TNF-α blocking agents bind with TNF-α molecules thereby neutralizing the biological activity of TNF-α, resulting in the reduction of intestinal inflammation.

On other hand, treatment strategies for fistulizing CD are usually controversial. External fistulas are more responsive to medical therapy than internal fistulas in patients with CD. Combined treatment with antibiotics and immunomodulators may be a suitable initial therapy for CD patients with external fistulas. TNF-α blocking agents can also be used as an additional therapy in the treatment of corticosteroid dependent, refractory cases or in complicated extraintestinal manifestations[10].

This case report demonstrates the potential efficacy of adalimumab in the management of complicated fistulizing CD and/or pyoderma gangrenosum. Further studies into the use of adalimumab in this patient subgroup with CD are warranted.

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CASE REPORT

Scirrhous hepatocellular carcinoma displaying atypical findings on imaging studies

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Abstract

We describe a 15-mm scirrhous hepatocellular carcinoma (HCC) in a 60-year-old man with B-type cirrhosis. Ultrasound disclosed a 15-mm hypoechoic nodule in segment 7. Contrast-enhanced US revealed heterogeneous, not diffuse, hypervascularity in the early phase and a defect in the Kupffer phase. Contrast-enhanced computed tomography (CT) revealed a heterogeneous hypervascular nodule in the early phase and a low-density area in the late phase. Magnetic resonance imaging (MRI) revealed iso- to hypointensity at T1 and high intensity at T2-weighted sequences. Contrast-enhanced MRI also revealed a heterogeneous hypervascular nodule in the early phase and washout in the late phase. Super-paramagnetic iron oxide-MRI revealed a hyperintense nodule. CT during hepatic arteriography and CT during arterial portography revealed heterogeneous hyperattenuation and a perfusion defect, respectively. Based on these imaging findings the nodule was diagnosed as a mixed well-differentiated and moderately-differentiated HCC. Histologically, the nodule was moderately-differentiated HCC characterized by typical cytological and structural atypia with dense fibrosis. Immunohistochemically, the nodule was positive for heterochromatin protein 1 and alpha-smooth muscle actin, and negative for cytokeratin 19. From the above findings, the nodule was diagnosed as scirrhous HCC. Clinicians engaged in hepatology should exercise caution with suspected scirrhous HCC when imaging studies reveal atypical findings, as shown in our case on the basis of chronic liver disease.

Key words: Scirrhous hepatocellular carcinoma; Contrast-enhanced computed tomography; Contrast-enhanced magnetic resonance imaging; Contrast-enhanced ultrasound; Computed tomography during hepatic arteriography; Computed tomography during arterial portography; Heterogeneous hypervascularity

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Kim SR, Imoto S, Nakajima T, Ando K, Mita K, Fukuda K, Nishikawa R, Koma Y, Matsuoka T, Kudo M, Hayashi Y. Scirrhous hepatocellular carcinoma displaying atypical findings on imaging studies. *World J Gastroenterol* 2009; 15(18): 2296-2299 Available from: URL: http://www.wjgnet.com/1007-9327/15/2296.asp DOI: http://dx.doi.org/10.3748/wjg.15.2296

INTRODUCTION

According to World Health Organization (WHO) classifications, hepatocellular carcinoma (HCC) with diffuse fibrosis is subclassified as scirrhous-type HCC (SHCC)[1]. Histologically, it is characterized by diffuse fibrosis along the sinusoid-like blood spaces with varying degrees of atrophy of tumor trabeculae. Preoperative images by computed tomography (CT) and magnetic resonance imaging (MRI) are, however,

often misdiagnosed as those of cholangiolocellular carcinoma (CCC), HCC-CCC, and metastatic carcinoma due to heterogeneous enhancement in the early phase and prolonged enhancement in the late phase attributed to abundant fibrous stroma. Moreover, imaging studies for the diagnosis of SHCC, such as contrast-enhanced ultrasound (US), CT during hepatic arteriography (CTA) and CT during arterial portography (CTAP) have so far not been described. Here, we present a case of moderately differentiated SHCC that histologically manifested as typical cytological and structural atypia with dense fibrosis, whereas imaging studies with contrast-enhanced CT, MRI, US, CTA and CTAP revealed a mixed well-differentiated and moderately-differentiated HCC.

CASE REPORT

A 60-year-old man with B-type liver cirrhosis was admitted in November 2007 for further examination of a 15-mm hypoechoic nodule in segment seven (S7). The patient had no history of alcohol, blood transfusion or drug abuse. On admission, physical examination showed no remarkable abnormalities. Hepatitis B virus was positive for surface antigen and for envelope antibody, and negative for envelope antigen (HBeAg). The amount of HBV deoxyribonucleic acid was less than 2.6 log copy/mL. Laboratory studies disclosed the following abnormal values: platelets $5.3 \times 10^4/\mu L$ (normal, 14-34), aspartate aminotransferase 44 IU/L (0-38), alkaline phosphokinase 864 IU/L (115-359), thymol turbidity 7.7 U (0-4), zinc sulfate turbidity test 14.8 U (2-12), and γ-globulin 29.3 g/dL (10.6-20.5). The levels of tumor markers were as follows: alpha-fetoprotein (AFP) 3.8 ng/mL (< 10), protein induced by vitamin K absence 71 mAU/mL (0-40), CA19-9 39.4 U/mL (0-37), and CEA 4.78 ng/mL (0-5).

US disclosed a 15-mm hypoechoic nodule in S7. Contrast-enhanced CT revealed a heterogeneous, not diffuse, hypervascular nodule in the early phase and a low-density area in the late phase (Figure 1A and B). MRI revealed iso- to hypointensity at T1 and high intensity at T2-weighted sequences. Contrast-enhanced MRI revealed a heterogeneous hypervascular nodule in the early phase and washout in the late phase (Figure 2A and B). Super-paramagnetic iron oxide-MRI revealed a hyperintense nodule. Contrast-enhanced US revealed heterogeneous hypervascularity in the early phase and a defect in the Kupffer phase (Figure 3A and B). CTA and CTAP revealed heterogeneous hyperattenuation and a perfusion defect, respectively (Figure 4). Based on these imaging findings, the nodule was diagnosed as a mixed well-differentiated and moderately differentiated HCC. Histologically, the nodule was moderately-differentiated HCC characterized by typical cytological and structural atypia with dense fibrosis (Figure 5A and B). Immunohistochemically, the nodule was positive for heterochromatin protein 1 and alpha-smooth muscle actin (α-SMA) (Figure 5C and D), and negative for cytokeratin 19 (CK19). From the above findings, the nodule was diagnosed as SHCC. We conducted radiofrequency ablation for the SHCC and the nodule was completely ablated. Local recurrence has not been observed over a period of 15 mo.

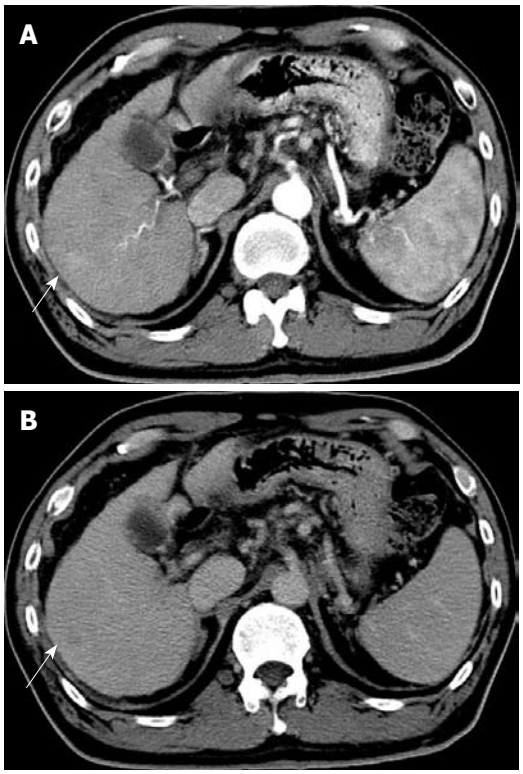

Figure 1 Contrast-enhanced CT. Heterogeneous, not diffuse, hypervascular nodule in the early phase (A) (arrow), and a low-density area in the late phase (B) (arrow).

DISCUSSION

The clinical background of SHCC is not significantly different from that of non-SHCC with regard to age, gender, positive rates to hepatitis viruses, AFP levels, Child-Pugh classification, and the stage of tumor-node-metastasis. In both morbidities, over 60% of cases are associated with chronic hepatitis rather than with liver cirrhosis. HCC patients with liver cirrhosis and liver dysfunction tend not to undergo surgery. In our case, resection was not carried out because of poor liver function attributed to liver cirrhosis.

With no clear pathological definition of SHCC, in particular a standard for the degree of the fibrosis for diagnosing the disease, its rate varies between 0.2% and 4.2%[2,3]. Regarding terminology, SHCC is often confused with "sclerosing hepatic carcinoma" that is used to designate a variety of tumors with sclerotic change and hypercalcemia arising in non-cirrhotic livers[4]. Sclerosing hepatic carcinoma does not, however, constitute a distinct histopathological entity; some of these tumors appear to be HCC, others CCC. Therefore, sclerosing hepatic carcinoma has been deleted from the WHO classification[1]. Kurogi *et al*[5] have defined SHCC as a tumor with diffuse fibrous changes in almost the entire area of the largest cross-section of the tumor and a mean fibrotic area of 39% compared with only 4.6%

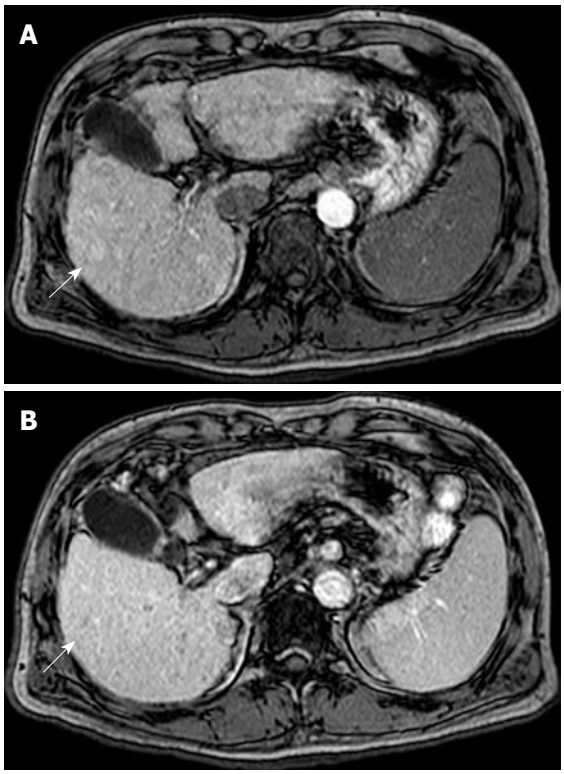

Figure 2 Contrast-enhanced MRI. A heterogeneous hypervascular nodule in the early phase (A) (arrow), and washout in the late phase (B) (arrow).

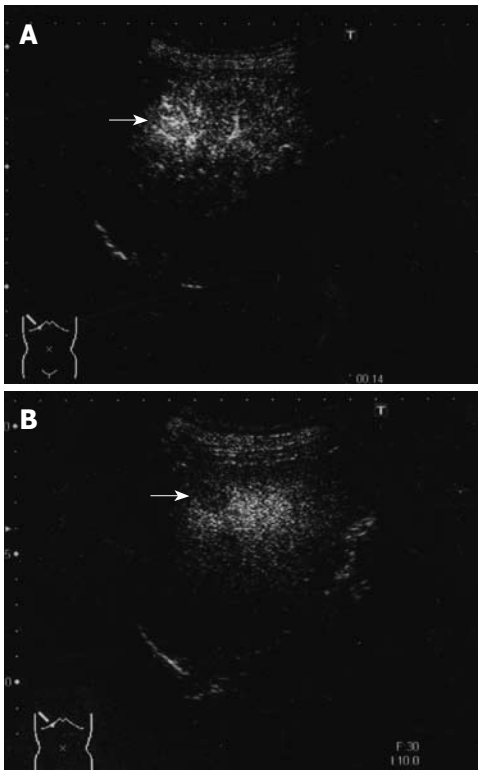

Figure 3 Contrast-enhanced US. Heterogeneous hypervascularity in the early phase (A) (arrow), and defect in the Kupffer phase (B) (arrow).

in non-SHCC.

SHCC is characterized by stellate fibrosis (84%), no encapsulation (absence of capsule) (100%), no necrosis and hemorrhage (100%), intratumoral portal tracts (80%), remarkable lymphocyte infiltration (84%), clear cell change (84%), and hyaline bodies (52%). The number of α-SMA-positive myofibroblast-like cells (activated stellate cells) in the tumor is about three times that in non-SHCC[5].

SHCC is occasionally misdiagnosed as fibrolamellar carcinoma (FLC) because of the presence of lamellar fibrosis. FLC is common in young adults and usually arises in the liver without any underlying chronic liver disease. Histologically, FLC is characterized by polyhedral, deeply eosinophilic neoplastic hepatocytes with round nuclei and distinct nucleoli, many of which contain intracytoplasmic hyaline globules and distinct pale bodies, and fibrosis arranged in a lamellar fashion around the neoplastic hepatocytes[6,7]. Conversely, although SHCC occasionally presents with lamellar fibrosis, the cancer cells being different from those of FLC, it is common in older patients with associated chronic hepatitis or liver cirrhosis[5]. Accordingly, it is not difficult to differentiate SHCC from FLC. In our case, the nodule was not diagnosed as FLC, clinically or histologically.

The US pattern was mostly hypoechoic, and contrast-enhanced CT and MRI revealed mostly heterogeneous hypervascularity in the early phase. The most characteristic feature of the imaging studies was prolonged enhancement in the late phase. Incidentally, imaging studies such as contrast-enhanced US, CTA and CTAP have, so far, not been described for use in the diagnosis of SHCC. Misdiagnosis by imaging studies is more frequent in SHCC than non-SHCC. Of 25 cases of SHCC, nine (36%) have been diagnosed as CCC, combined HCC-CCC, and metastatic carcinoma characterized by abundant fibrous stroma, the misdiagnosis being attributed to the prolonged enhancement of the tumor in the late phase and heterogeneous enhancement in the arterial phase on contrast-enhanced CT[5].

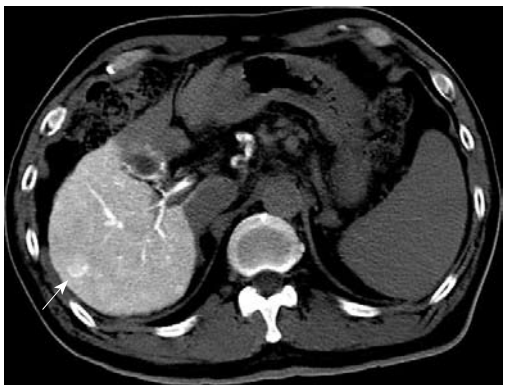

Figure 4 Heterogeneous hyperattenuation at CTA (arrow).

In our case, contrast-enhanced CT, MRI, US revealed heterogeneous hypervascularity in the early phase; the nodule was not misdiagnosed as CCC or HCC-CCC because the imaging findings showed no prolonged enhancement in the late phase. The nodule was misdiagnosed as well-differentiated and moderately-

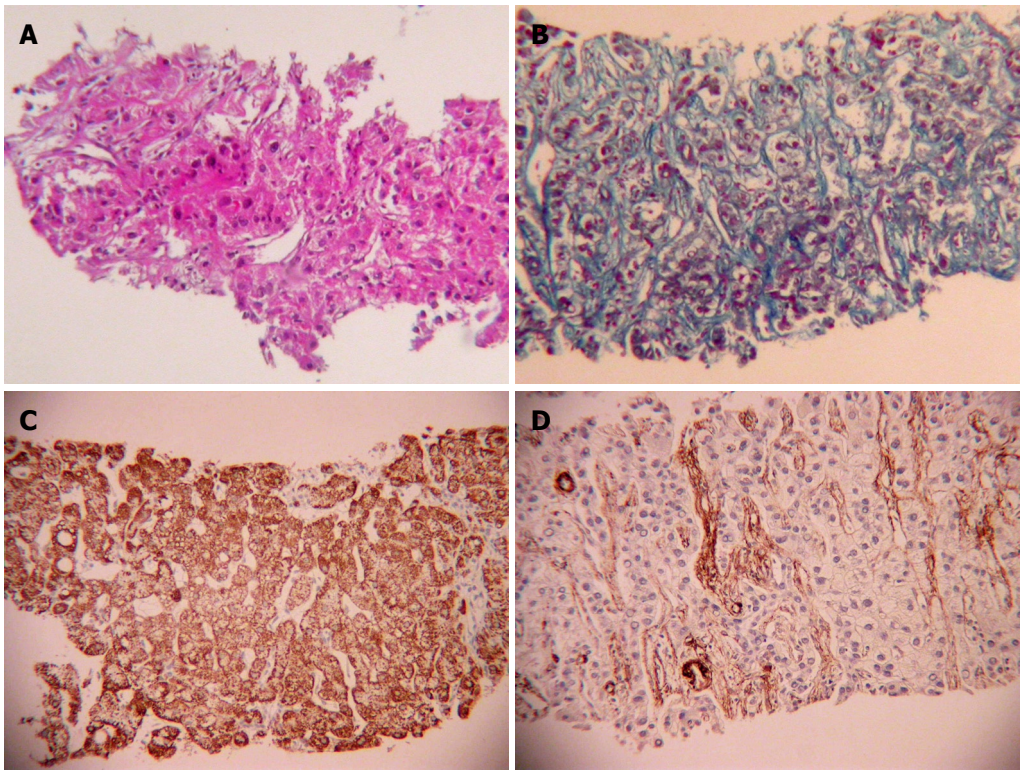

Figure 5 US-guided biopsy. Moderately-differentiated HCC characterized by typical cytological and structural atypia with dense fibrosis (HE stain) (A), (Mallory-Azan stain) (B). Positive for Hp1 (C) and α-SMA (D).

differentiated HCC on contrast-enhanced CT, MRI and US, which showed heterogeneous hypervascularity in the early phase and washout in the late phase; CTA and CTAP showed heterogeneous hypervascularity in the arterial and perfusion defect in the portal phases. Immunohistochemically, the nodule was negative for CK19 and, therefore, not CCC. Although contrast-enhanced US, CTA and CTAP did not indicate SHCC, these modalities are very effective in showing the heterogeneous vascular component, washout and perfusion defect of the nodule and contribute to precise diagnosis.

Clinicians engaged in hepatology should exercise caution with suspected SHCC when imaging studies reveal atypical findings, as shown in our case on the basis of chronic liver disease.

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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
London, UK
Gastro 2009 UEGW/World Congress of Gastroenterology
www.gastro2009.org

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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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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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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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EDITORIAL

Hepatitis C virus lymphotropism and peculiar immunological phenotype: Effects on natural history and antiviral therapy

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Abstract

Hepatitis C virus (HCV) has been recognized to be both a hepato- and lymphotropic virus. HCV lymphotropism represents an essential lap in the pathogenesis of virus-related autoimmune and lymphoproliferative disorders, ranging from clonal expansion of B-cells with organ- and non-organ-specific autoantibody production up to overt non-Hodgkin's lymphoma along a continuous step-by-step model of B-cell lymphomagenesis, where the intermediated mixed cryoglobulinemia could be considered as a stage of suppressible antigen-driven lymphoproliferation. HCV infection of lymphoid cells could set up privileged reservoirs able to interfere with the host viral clearance efficiency and may be implicated in viral recurrence after apparently successful antiviral therapy. The HCV long-lasting extrahepatic replicative state generates an abnormal systemic immunological response, easily detectable by searching simple laboratory and clinical parameters, mainly represented by vasculitis-like skin features and hypocomplementemia. The presence or absence of this hypersensitivity pattern seems to correlate with the antiviral response and could be identified as a novel immunological cofactor. Further research is required to fully verify the real impact on therapeutic choice/regimen.

Key words: Hepatitis C virus; Lymphotropism; Natural history; Antiviral therapy; Immunological co-factor

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INTRODUCTION

Hepatitis C virus (HCV) infection has been currently identified as the leading cause of chronic liver disease, including cirrhosis and hepatocellular carcinoma, in Western countries. However, despite its large diffusion (with over 170 million of people infected world-wide), the lack of symptoms during the acute phase, together with the indolent course of the disease over time, hampers the difficulties to assess the natural history of the disease. This complexity can also be argued from the wide heterogeneity of disease complications' rate observed when different methodological approaches were used. Moreover, the progression of the disease could also be dramatically affected by many variables related to the host, the virus and the environment. The global socioeconomic burden of HCV is magnified by hundreds of thousands of infections identified every year. Finally, in the last few years, the long-term outcome of the infected subjects has been deeply modified by the use of efficient antiviral therapy.

HCV has been recognized to be both hepato- and lymphotropic virus; HCV lymphotropism represents an essential lap in the pathogenesis of virus-related immunological disorders[1], being responsible for poly-oligoclonal expansion and consequent wide organ- and non-organ-specific autoantibody production, including rheumatoid factor (RF) and cryo- and non-cryoprecipitable immune complexes.

IMMUNOLOGICAL MECHANISMS

HCV, belonging to the Flaviviridae family, is a positive single-stranded RNA virus, without a DNA intermediate of replication, unable to integrate into the host genome[2].

Otherwise, HCV affects cellular functions modulating the immune response, cell proliferation or apoptosis, so facilitating the clonal B-lymphocyte spread[3-5]. HCV may exert an antigen-driven chronic stimulus on the immune system through several viral proteins[6]. An important pathogenetic step is the interaction between the HCV envelope protein E2 and the CD81 molecule, a fairly ubiquitary tetraspanin present on both hepatocytes and B-cells surface[7], ending up to a strong and sustained polyclonal stimulation of B-cell compartment. CD81 is a part, on the B-cell, of a complex with CD21, CD19 and Leu 13; such complex lowers the threshold for B-cell activation by bridging antigen-specific recognition and CD21-mediated complement recognition[8]. Then, the interaction between HCV-E2 and CD81 could increase the frequency of VDJ rearrangement in antigen reactive B-cells[4,5,9], with possible bcl-2 proto-oncogene activation[4,5]. Again, the latter stage could be secondary to t(14; 18) translocation alone, repeatedly observed in B-cells of HCV-infected individuals, particularly in those with type Ⅱ mixed cryoglobulinemia (MC)[4,6]. Bcl-2 proto-oncogene is able to inhibit apoptosis, leading to abnormal B-cell survival[6]. Besides, the prolonged B-cell survival could prompt, in presence of additional factors (genetic, epigenetic, hormonal and immunological), other genetic aberrations up to overt non-Hodgkin's lymphoma (NHL), as late complication of the MC syndrome[1,9]. The critical question remains whether HCV replication occurs in normal B-cells and is directly lymphomagenic or is lymphomagenesis a stochastic process accompanying HCV-driven proliferation of B-cells[10]. At all, given its biological characteristics, HCV may be involved in several autoimmune and lymphoproliferative disorders, and the multifaceted HCV syndrome can fit in a continuous step-by-step model of B-cell lymphomagenesis, whereby MC could be viewed as marker of antigen-driven lymphoproliferation and frank NHL as loss of antigen-dependence[11].

CLINICAL IMPLICATIONS

At this point, the most scheming and open issue is the definite weight placed on the HCV natural history by its lymphotropism. Firstly, HCV infection of lymphoid cells could condition HCV persistence. In fact, lymphoid cells, and particularly long-living subsets and/or bone-marrow elements, may represent privileged reservoirs able to interfere with host viral clearance efficiency, by impairing the capability of immune response and/or by facilitating selection of distinctive viral variants[12]. Nowadays, it remains indefinite how infection of the immune cells by HCV may alter their functions, although impairment in the allostimulatory capability of HCV-infected dendritic cells derived from patients with chronic hepatitis C has been reported[13]. Interestingly, the entity of this extrahepatic reservoir seems to be correlated with the length of infection[12] and may be implicated in HCV recurrence after apparently successful antiviral therapy[1], the peripheral blood mononuclear cells (PBMC) being a potential viral tank resistant to interferon (IFN). In fact, in several researches PBMC infection appeared as a negative factor of patient on-IFN response[14-18] or predictive for relapse off-IFN monotherapy[15,18-20] or -combined (plus ribavirin) antiviral treatment[21]; on the other hand, such prognostic value was not observed in other studies and is still a controversial question[22-25]. Previous literature data report sustained response (SR) in a near 10% of patients with chronic HCV infection after IFN alone. Classical predictors of response include viral load and genotype, as well as histological (fibrosis score) and metabolic[26] features or alcohol as cofactor. Also the immunological background is associated with antiviral response, the cellular immune functions being essential to the elimination of HCV-infected hepatocytes. A basal low T-helper type 1 and type 2 ratio predicted a higher SR rate in a Japanese cohort[27]. Nevertheless, the immunological pattern remains poorly explored. Conflicting data have been reported on the prevalence of MC in chronic HCV patients, ranging from less than 5% up to 50 %[28,29]. In most cases type M immunoglobulins with RF activity have been found in cryoprecipitates[30], inducing the deposition of immune complexes in small vessels (vasculitis)[31]. The MC-related clinical manifestations, including purpura, arthralgias and weakness, and complications, as glomerulonephritis, neuropathic lesions, B-cell NHL, reflect a systemic involvement that may lessen the chance of viral eradication. In a recent effort[32], it was retrospectively confirmed that this immunological phenotype, also labelled as type Ⅲ or hypersensitivity disorder, is significantly associated with a higher risk of viral persistence after IFN monotherapy, with skin involvement and hypocomplementemia being independent predictors of lack of response; conversely, this study suggested that in the absence of common negative predictors, such as this last immunological cofactor, SR could be reached also by a therapeutic approach based on IFN monotherapy. What practical implications does HCV lymphotropism suggest? No factor is currently available to predict the productive HCV infection of PBMC, but such event is likely to be time-dependent during the natural history of HCV infection. In other words, we observe the serological response and the clinical effects of a long-lasting extrahepatic replicative state, i.e. an abnormal immunological status. This picture can be easily detected by looking for cryoglobulins, RF, antinuclear antibody, complement fractions, circulating immune complexes (C1q protein and C1q binding), mono-oligoclonal gamma-globulin expansion or vasculitis-related clinical manifestations including skin lesions (palpable purpura or hyperpigmented macule of the lower limbs), sensory-motor peripheral neuropathy (gait impairment associated with paresthesia and cramps), arthralgias, as well as urinary changes suggestive of glomerular derangement, i.e. microalbuminuria (in the absence of hypertension and diabetes), all variously combined (at least four out of the above mentioned laboratory and clinical parameters)[32]. This approach is reliable and less expensive or hard than direct detection of HCV in PBMC. The antiviral therapy (IFN plus ribavirin) significantly counteracts the

exaggerated immune response, also independently from the viral outcome[33], through different mechanisms: IFN could affect the intrahepatic T-cell response and inhibit interleukin (IL) 10 production[34], meanwhile ribavirin suppresses IL 10, IL 12 and tumor necrosis factor-alpha[35]; since the hypersensitivity disorders are the expression of a polyclonal activation of B-cells, due to stimulation by T-cells, it could be hypothesized that the changes induced by the combined therapy in the cytokine pattern determine a down-regulation of the mechanism of stimulation T-cells/B-cells. Sometimes, polyclonal B-cell hyperactivity partially escapes from the immune modulation effects of the antiviral treatment, so that the immunological spectrum persists after HCV clearance and suppression of the antigenic stimulus[33]. On the other hand, the undetectability of serum HCV RNA does not mean complete viral clearance, since genomic material has been found in PBMC of SR patients[12], and, therefore, an ongoing immune-stimulation cannot be excluded; interestingly, in those same patients with occult PBMC infection, a persistence of the MC syndrome, even if to a lesser degree with respect to the pre-treatment period, was observed, suggesting a potential advantage of using a prolonged course of antiviral treatment to obtain a sufficient consolidation. The discovery of occult HCV infection has challenged the paradigm that apparent complete resolution of hepatitis C, either spontaneously or therapeutically-induced, would be indicative of eradication of HCV[36]; persistent HCV replication in hepatocytes and PBMC would likely drive the continuous antigenic stimulation of the immune system in immunocompetent patients, which, in turn, allows the host to keep this silent infection under relative control[37]; again, such prolonged HCV replication associated with the chronic presentation of HCV antigens by infected B-cells and monocytes could contribute to the immune tolerance of HCV, thus supporting even further HCV persistence[37]. Finally, negativization of anti-HCV antibody, unrelated to an immunosuppression context, occurs in a percentage of long-term (on average 4 years) SR patients showing a weaker CD4+-specific HCV reactivity; such lessened immunological spur could mirror a full disappearance of the minimal, residual viral amounts, although the potential localization of a further load within PBMC, without release of viral particles into the serum, cannot be ruled out[38]. Only a prolonged follow-up will be able to verify the definitive clearance.

In conclusion, the treatment of hepatitis C is expensive, often demanding, from the patients' perspective, and difficult as far as the decision about whom, when and for how long to treat. Another predictor of response to antivirals is recognized, as immunological cofactor, i.e. the HCV lymphotropism. Further studies are warranted to evaluate alternative antiviral schedules depending on the presence or the absence of this additional cofactor.

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EDITORIAL

Clinicopathological features of early gastric cancer with duodenal invasion

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Abstract

The incidence of early gastric cancer (EGC) with duodenal invasion is extremely low, although advanced gastric cancer that arises in the antrum occasionally invades the duodenum. We investigated the clinicopathological features of EGC with duodenal invasion and provided strategies for clinical management. A Medline search was performed using the keyword "early gastric cancer" and "duodenal invasion". Additional articles were obtained from references within the papers identified by the Medline search. We revealed that EGC with duodenal invasion was of the superficial spreading type of tumor. Tumors > 60 mm in size invaded the duodenum more extensively, and the distance of duodenal invasion from the pyloric ring was further in the elevated type than in the depressed type of tumor. There was no significant difference between the length of duodenal invasion and the histological type of the tumor. Gastric cancer located adjacent to the pyloric ring, even if cancer invasion was confined to the mucosa or submucosa, was more likely to invade the duodenum. The present study reveals that the elevated type of EGC is associated with more extensive duodenal invasion when the tumor size is > 60 mm, thus highlighting the importance of identification of duodenal invasion in these cases. We also reveal that sufficient duodenal resection with a cancer-free distal surgical margin should be performed in cases of duodenal invasion.

Key words: Duodenal invasion; Early gastric cancer; Gastrectomy; Superficial spreading type; Tumor differentiation

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INTRODUCTION

Early gastric cancer (EGC), which is defined as a lesion confined to the mucosa or the submucosa, regardless of the presence of lymph node metastasis, has a good prognosis with surgical treatment. However, a small number of patients experience recurrence of EGC after resection. Sano *et al*[1] have reported that, in a study of 1475 patients with EGC treated with surgery, 1.4% experienced disease recurrence. The incidence of recurrence of EGC was shown to be significantly higher in the patient group with submucosal, node-positive and undifferentiated tumors. Furthermore, some rare cases show distant metastasis, such as in liver, lung, or bone, even though the depth of cancer invasion is confined to the mucosa[2]. Sufficient resection margins are necessary to prevent recurrence of EGC, because inadequate resection that does not maintain surgical margins free of cancer can lead to disease recurrence. Duodenal invasion by gastric cancer is encountered in 11.9%-23.8% of all patients with cancer in the gastric antrum[3-5]. However, EGC with duodenal invasion is rare amongst cases of advanced gastric cancer[6]. There have been very few case reports of this type of cancer. Since the literature on this subject consists mostly of isolated case reports, the clinicopathological features of EGC with duodenal invasion remain unclear. We attempted to elucidate the clinicopathological features of patients with EGC extended to the duodenum, and discuss the possible mechanisms underlying this rare condition and practical surgical strategies.

PATIENTS AND CLINICOPATHOLOGICAL PRESENTATION

We reviewed 41 patients who underwent surgical resection for EGC with duodenal invasion between

Table 1 Clinicopathological data for 41 cases of EGC with duodenal invasion

Authors	Year	Age (yr)	Gender	Location	Type	Size (mm)	Depth of invasion	Lymph node metastasis	Histological type	Distance of duodenal invasion (mm)	Preoperative diagnosis
Ishii	1975	50	M	Circ	Depressed	32 × 25	m	-	Intestinal	7	ND
		47	M	Less	Elevated	30 × 15	sm	-	Intestinal	5	ND
Kuwayama[15]	1976	72	M	Ant-Less	Depressed	40 × 35	m	-	Diffuse	4	Impossible
Uchida[13]	1979	50	M	Circ	Depressed	32 × 25	m	-	Diffuse	7	ND
		61	M	ND	Mixed	35 × 21	sm	-	Intestinal	2	ND
		47	M	ND	Elevated	30 × 15	sm	-	Intestinal	5	ND
Kuwata[18]	1981	ND	ND	Less	Depressed	ND	sm	-	Diffuse	5	Impossible
		ND	ND	Gre	Elevated	ND	sm	-	Intestinal	6	Impossible
		ND	ND	Less	Elevated	ND	sm	-	Intestinal	2	Impossible
		ND	ND	Post	Mixed	ND	sm	-	Intestinal	1	Impossible
		ND	ND	Less	Mixed	30 × 15	sm	-	Intestinal	2	Impossible
		ND	ND	Less	Depressed	35 × 21	sm	-	Intestinal	1	Impossible
		67	M	Less	Mixed	30 × 15	sm	-	Intestinal	5	Impossible
Kato[17]	1993	63	F	Circ	Elevated	68 × 38	m	-	Intestinal	16	Possible
Nakazawa[9]	1994	58	M	Ant-Gre	Depressed	10 × 10	sm	-	Intestinal	3	Impossible
Boku[27]	1996	73	F	Circ	Elevated	70	sm	-	Intestinal	35	Possible
Ito[14]	1996	76	F	Less	Mixed	45 × 35	sm	-	Intestinal	25	Possible
Matsumoto[16]	2000	ND	ND	Less	Elevated	25 × 9	m	ND	Diffuse	3	Impossible
		61	M	Gre-Post	Elevated	25 × 10	sm	ND	Diffuse	10	Possible
		ND	ND	ND	Elevated	30 × 13	m	ND	Intestinal	3	Impossible
		ND	ND	ND	Elevated	65 × 23	m	ND	Intestinal	3	Impossible
		ND	ND	ND	Superficial	45 × 45	sm	ND	Intestinal	5	Impossible
Nogueira[12]	2000	ND	ND	ND	ND	ND	m	-	Diffuse	3	ND
		ND	ND	ND	ND	ND	sm	-	Intestinal	7	ND
Yasuda[23]	2000	59	F	Circ	Depressed	72 × 15	m	-	Diffuse	11	Impossible
Nakayama[37]	2001	59	F	Circ	Mixed	85 × 75	sm	-	Intestinal	38	Possible
Koufuji[29]	2003	77	M	Circ	Mixed	70 × 50	sm	-	Intestinal	2	ND
		65	M	Circ	Elevated	90 × 55	sm	-	Intestinal	2	ND
		66	F	Circ	Depressed	120 × 98	m	-	Diffuse	2	ND
		70	F	Circ	Depressed	130 × 102	m	-	Diffuse	8	ND
		58	M	Less	Depressed	55 × 24	sm	+	Diffuse	3	ND
		81	F	Gre	Depressed	30 × 20	sm	+	Intestinal	2	ND
		44	M	Less	Depressed	52 × 30	sm	-	Intestinal	5	ND
		57	F	Circ	Depressed	57 × 33	m	-	Diffuse	3	ND
		68	F	Gre	Depressed	40 × 38	sm	+	Diffuse	5	ND
		58	F	Circ	Depressed	80 × 65	sm	+	Diffuse	2	ND
Ishikawa[24]	2005	72	M	Less-Post	Elevated	68 × 37	m	-	Intestinal	20	Possible
Matsuda[6]	2007	79	F	Circ	Elevated	30 × 15	sm	-	Intestinal	12	Possible
Our case	2008	49	M	Less	Depressed	30 × 12	m	-	Intestinal	1	Impossible
		63	M	Less	Elevated	35 × 15	m	-	Intestinal	3	Impossible
		84	F	Circ	Elevated	85 × 80	m	-	Intestinal	38	Possible

F: Female; M: Male; ND: Not described; Circ: Circumferential; Less: Lesser curvature; Gre: Greater curvature; Ant: Anterior; Post: Posterior; Intestinal: Papillary and tubular adenocarcinomas; m: Mucosa; sm: Submucosa; Diffuse: Poorly differentiated adenocarcinoma, signet ring cell carcinoma, and mucinous adenocarcinoma.

1975 and 2008. Thirty-eight cases were identified in the available literature using a Medline search and Japan Centra Revuo Medicina by use of the keywords "early gastric cancer" and "duodenal invasion". Additional articles were obtained from references within the papers identified by the searches. Three cases were patients treated in our hospital. Data on age, gender, tumor location, tumor type, tumor size, depth of invasion, lymph node metastasis, histological type, and preoperative diagnosis of duodenal invasion for each patient were obtained. The clinicopathological features of the 41 reported cases are listed in Table 1. Of the 41 patients analyzed, the mean age of patients was 63.2 years (range, 44-84 years), and there was a slight male predominance, with a male-to-female ratio of 16:13. The average diameter of tumors was 51.6 mm (range, 10-130 mm). The average distance of duodenal invasion was 7.9 mm (range, 1.0-38 mm). The case with the maximal distance of duodenal invasion was one of our cases. All patients had undergone curative tumor resection. There was no lymph node metastasis in cases in which the tumor was confined to the mucosa, whereas of the 25 patients in which the tumor had invaded the submucosa, four had lymph node metastasis. There was no lymphatic or venous invasion or distant metastasis.

The Mann-Whitney *U* test was used to assess correlations among the mean values for each group. The Pearson χ^2 test was applied to qualitative variables. All values are expressed as mean ± SD. $P < 0.05$ was considered significant.

EFFECT OF TUMOR INVASION DISTANCE

Table 2 shows the results of univariate analysis of the distance of duodenal invasion from the pyloric ring in relation to eight selected variables: age, gender, gross appearance, tumor size, depth of invasion, histological type, lymph node metastasis, and preoperative diagnosis

Table 2 Clinicopathological characteristics of EGC with duodenal invasion

Characteristics	No. of patients	Length of duodenal invasion (mm)	*P* value
Age (yr)			0.276
< 60	13	7.2	
> 60	16	11.7	
Gender			0.029
Male	16	5.3	
Female	13	15.2	
Gross appearance			0.046
Depressed	16	4.3	
Elevated	15	10.9	
Tumor size (mm)			0.049
< 60	23	5.3	
> 60	12	14.8	
Depth of invasion			0.836
Mucosa	16	8.3	
Submucosa	25	7.6	
Histological type			0.088
Intestinal	28	9.1	
Diffuse	13	5.1	
Lymph node metastasis			0.006
Negative	32	8.9	
Positive	4	3	
Preoperative diagnosis of duodenal invasion			0.001
Possible	8	24.3	
Impossible	16	3.6	

of duodenal invasion. The distance of duodenal invasion by EGC was 4.5 mm for depressed type tumors, 11.4 mm for elevated type tumors, 5.3 mm for tumors with a diameter < 60 mm, and 14.8 mm for tumors with a diameter > 60 mm. These results revealed a positive correlation between more extensive duodenal invasion and elevated type tumors with a size > 60 mm.

In advanced gastric cancer, the rate of metastasis to the lymph nodes was high when the distance of duodenal invasion was > 10 mm[7]. By comparison, we found lymph node metastasis in only four cases of EGC, and in each of these, invasion had reached the submucosa and the distance of duodenal invasion was < 10 mm. This result suggests that there is a strong positive correlation between the incidence of lymph node metastasis and submucosal invasion, regardless of the distance of duodenal invasion.

PREOPERATIVE DIAGNOSIS OF EGC WITH DUODENAL INVASION

Generally, preoperative diagnosis of malignant invasion to the duodenum is difficult[8,9], because spread of gastric cancer to the duodenum is often infiltrative and invades directly through the submucosal or subserosal layer[10-12]. Most of these cases are advanced gastric cancer[13]. In EGC, gastroenteroscopic examination is a reliable technique for identifying the area of cancer infiltration[14]. It is necessary to accurately define the tumor margin in order to determine the resection line. However, it is occasionally difficult to accurately determine the margin of the tumor in the vicinity of the pyloric ring by endoscopy[15-17]. This is because the pyloric ring is a narrow lumen, making it difficult to observe the tumor, and it can be deformed by ulcers, mucosal atrophy, and metaplastic changes. Moreover, pyloric movement caused by strong peristalsis and reflux of bile prevent the satisfactory observation of the lesion on the pyloric ring[15].

Duodenal invasion by EGC was diagnosed preoperatively by esophagogastroduodenoscopy (EGD) or barium meal examination in only eight cases (Table 1). The mean distance of duodenal invasion was 24.3 mm in the group in which a preoperative diagnosis was possible, whereas it was 3.6 mm in the group in which a preoperative diagnosis was not possible. There was a significant difference between the two groups (Table 2). In these cases, the distance of duodenal invasion was greater for elevated or mixed type tumors > 10 mm in diameter. Of the nine cases in which the distance of duodenal invasion was > 10 mm, there was only one case in which a preoperative diagnosis of duodenal invasion was not possible. By comparison, no case could be diagnosed preoperatively where the distance of duodenal invasion was < 10 mm. These results suggest that a preoperative diagnosis of duodenal invasion is related to tumor type and size. Kuwata *et al*[18] have reported that radiological diagnosis of duodenal invasion is more useful in the elevated type than in the depressed type of tumor, and that the compression method gives a more accurate diagnosis than the double-contrast method. Furthermore, despite extensive preoperative examination, determination of the tumor margin is often not possible in patients with a superficial spreading type of gastric cancer[19-22]. Thus, a satisfactorily precise diagnostic approach to assess the extent of tumor invasion has not been established.

MECHANISMS OF DUODENAL INVASION BY EGC

The border between the stomach and the duodenum is not clinically obvious. Brunner's glands can be considered as the start of the duodenum for the clinicopathological assessment of duodenal invasion by gastric cancer[3,13]. When gastric cancer directly invades the mucosal layer, the Brunner's glands remain intact, even when surrounded by cancer cells[3]. For this reason, it is thought that Brunner's glands prevent direct cancer invasion from the gastric mucosa to the duodenal mucosa. In a study of 141 patients with gastric carcinoma with duodenal invasion, there was only one case of intramucosal carcinoma[3]. In the case of a lesion caused by an ulcer, it is speculated that destruction of the mucosal structure of the duodenum by an ulcer located in the pylorus allowed gastric cancer to invade the duodenum[23]. In another case in which endoscopic mucosal resection (EMR) had been performed previously for gastric cancer in the area of the pyloric ring, it is thought that destruction of the gastroduodenal mucosal microanatomy by EMR allowed carcinoma cells to invade the duodenal mucosa[24].

The superficial spreading type of EGC is characterized by wide and superficial spreading activity of the

Table 3 Clinicopathological characteristics of EGC with duodenal invasion for superficial spreading and small-sized types

Characteristics	Superficial spreading type	Small-sized type	*P* value
Number of cases (%)	10 (27.0)	27 (73.0)	
Age (yr)	68.7 ± 8.1	60.4 ± 11.2	0.031
Gender			0.048
Male	3	13	
Female	7	6	
Gross appearance			0.281
Depressed	3	12	
Elevated	5	8	
Depth of invasion			0.614
Mucosa	5	11	
Submucosa	5	16	
Histological type			0.847
Intestinal	7	18	
Diffuse	3	9	
Lymph node metastasis			0.773
Negative	9	19	
Positive	1	3	
Length of duodenal invasion (mm)	16.3	5.4	0.044
Preoperative diagnosis of duodenal invasion			0.003
Possible	5	3	
Impossible	0	12	

cancer compared with a more limited depth of vertical invasion[25]. According to Yasui *et al*[26], EGC is classified as a superficial spreading type of tumor when the product of the longest diameter of the tumor and the diameter perpendicular to it is > 25 cm^2. Our study has revealed that gastric cancer with duodenal invasion is most often the superficial spreading type. Relations between the superficial spreading tumor and duodenal invasion of EGC may refer to multiple occurrence of cancer[27]. Previous authors have reported that the superficial spreading type accounted for 5.46%-11.0% of all EGC[19-22], whereas it accounted for 27.0% of EGC cases with duodenal invasion (Table 3). Duodenal invasion was more extensive in superficial spreading cancer lesions (16.3 mm) than in small-sized cancer lesions (5.4 mm). In both of these groups, there was no significant difference in the gross appearance, depth of invasion, histological type, or the incidence of lymph node metastasis. Taken together, these results suggest that the superficial spreading type of gastric cancer adjacent to the pyloric ring may have the potential to invade the duodenum.

STRATEGY FOR SURGICAL TREATMENT OF EGC WITH DUODENAL INVASION

The outcome of surgical treatment for EGC is generally considered to be satisfactory[1,28]. If EGC is treated with the appropriate surgical strategy, the outcome of treatment is excellent, even in patients with duodenal invasion[29]. However, Kakeji *et al*[30] analyzed 95 patients with duodenal invasion by gastric cancer, including advanced cases, and found that tumor spread into the duodenum was limited to within 2 cm in 76% of the patients and to within 3 cm in 81% of the patients. Therefore, for patients with advanced gastric cancer with duodenal invasion, gastrectomy with resection of 3-4 cm of the duodenum and sufficient lymph node dissection is recommended.

Recent advances in endoscopic and laparoscopic surgery now offer a better quality of life to patients with EGC[31]. Although the 5-year survival rate for EGC is ⩾ 90%[32], complete surgical extirpation of gastric cancer with a sufficient resection margin from the tumor, and removal of metastatic lymph nodes, is necessary for good prognosis in all EGC cases, including those with duodenal invasion[1,29,31,32]. Previous reports have revealed that the prognosis of gastric cancer patients is affected mostly by depth of invasion, followed by lymph node metastasis and tumor location[33,34]. Tumor size in gastric cancer is a reliable prognostic factor that might be a suitable candidate for use in the staging system[35]. However, tumor size is not an independent prognostic factor[36]. Tumor diameter > 3.5 cm has been identified as an independent factor for the occurrence of lymph node metastasis[33]. Our review revealed that many cases of EGC with duodenal invasion had larger tumors, with an average diameter of 51.6 mm, than cases without duodenal invasion. Among the cases of EGC with duodenal invasion, there was no cancer recurrence because suitable surgical resection had been performed. It is necessary for surgeons to identify a suitable resection line for the distal margin for preoperative diagnosis of duodenal invasion. In cases in which there is further extension of the tumor toward the duodenum, it may be necessary to determine a resection line using intraoperative EGD[37].

The indistinct tumor margins characteristic of superficial spreading tumors in EGC can lead to discrepancies in tumor area between surgical findings and pathological diagnosis[20,22]. Kasakura *et al*[19] have reported that, despite extensive preoperative examination, determination of the tumor margin was not possible in 26 of 59 patients with superficial spreading cancer. Furthermore, the number of metastatic lymph nodes was greater than with the common tumor type[21]. Accordingly, gastrectomy with extensive lymph node dissection with wide and sufficient surgical margin seems to be a most appropriate treatment for the superficial spreading type of EGC, including those cases with duodenal invasion. Based on these findings, treatment of superficial spreading type EGC, in which the distal margin is near the pyloric ring, should focus on attaining a satisfactory margin from the tumor.

CONCLUSION

Gastric cancer located adjacent to the pyloric ring, even if cancer invasion is confined to the mucosal or submucosal layer, has the potential for duodenal invasion, and surgeons should be aware of this possibility. The present study indicates that EGC of the elevated type with a tumor size > 60 mm correlates positively with more extensive duodenal invasion. Our findings highlight the importance of identification of duodenal invasion by pre- and intra-operative closed observation, and reveal that the resection line in cases of duodenal invasion should be performed with a cancer-free margin.

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TOPIC HIGHLIGHT

Yusuf Bayraktar, Professor, *Series Editor*

Non-classical phenotypes of autoimmune hepatitis and advances in diagnosis and treatment

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Abstract

Non-classical manifestations of autoimmune hepatitis can delay diagnosis and treatment. Our aims were to describe the clinical phenotypes that can confound the diagnosis, detail scoring systems that can ensure their recognition, and outline advances in treatment that can improve their outcome. Prime source and review articles in English were selected through Medline from 1970-2008 and assimilated into personal libraries spanning 32 years. Acute severe or asymptomatic presentations and atypical histological findings, including centrilobular zone 3 necrosis and concurrent bile duct changes, are compatible with the diagnosis. Cholangiographic abnormalities may be present in children and adults with the disease, and autoimmune hepatitis must be considered in patients without autoantibodies or with antimitochondrial antibodies and no other cholestatic features. Asymptomatic patients frequently become symptomatic; mild disease can progress; and there are no confident indices that justify withholding treatment. Two diagnostic scoring systems with complementary virtues have been developed to evaluate patients with confusing features. Normal liver tests and tissue constitute the optimal end point of treatment, and the first relapse is an indication for long-term azathioprine therapy. Cyclosporine, tacrolimus and mycophenolate mofetil are promising salvage therapies, and budesonide with azathioprine may be a superior frontline treatment. We conclude that the non-classical phenotypes of autoimmune hepatitis can be recognized promptly, diagnosed accurately, and treated effectively.

Key words: Non-classical phenotypes; Scoring systems; Treatment strategies

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INTRODUCTION

Autoimmune hepatitis was initially perceived as a self-perpetuating, inflammatory liver disease in young amenorrheic women with hirsutism, acne and cirrhosis[1-3]. The validity of this classical phenotype was subsequently strengthened by technological advances that excluded a viral cause for the condition[4] and by studies that implicated perturbations of the immune system in its pathogenesis[5-8]. The clinical phenotype expanded as the concept of autoimmunity was applied broadly to liver diseases of unknown cause and as the requirement for 6 mo of disease activity was eliminated from its definition[9,10]. Autoimmune hepatitis still lacks an etiological agent and disease-specific laboratory test, but its designation now applies to patient populations far more diverse and numerous than the original patients with "lupoid hepatitis"[11].

Autoimmune hepatitis must be considered in all individuals with acute and chronic hepatitis of undetermined cause and with graft dysfunction after liver transplantation[11]. Acute[12-15], acute severe[16-19],

and asymptomatic[20-22] forms have been described; progression to cirrhosis may be indolent and unsuspected[23-25]; transitions between active and inactive disease may occur spontaneously[26,27]; concurrent immune diseases may obscure the diagnosis[28,29]; serological markers may be variably expressed and absent at presentation[30-32]; histological findings may include centrilobular zone 3 necrosis[33-37] or concurrent biliary changes[38-42]; and different ethnic groups may have non-classical clinical phenotypes[43-47]. The identification of autoimmune hepatitis in diverse clinical situations is critical since prompt institution of corticosteroid therapy can be life-saving[48-50].

Corticosteroids alone or a lower dose in combination with azathioprine induce clinical, laboratory and histological improvement in 80% of patients within 3 years[51,52]. Ten- and twenty-year survivals exceed 80%[24], and hepatic fibrosis is reduced or prevented[53-56]. These therapeutic successes are counterbalanced against adverse outcomes that justify the continued pursuit of new drugs and regimens. Nine percent of treated patients deteriorate despite compliance with corticosteroid schedules (treatment failure)[57,58]; 13% develop treatment-related side effects that compel premature withdrawal of medication (drug toxicity)[59]; and 9% improve but not to a degree to justify discontinuation of the medication (incomplete response)[59]. Furthermore, 50%-86% of patients who enter remission relapse after drug withdrawal and require re-treatment[27,51,60-64].

The diagnostic criteria of autoimmune hepatitis have been codified by an international panel[9,10], and scoring systems can establish the diagnosis in difficult cases[9,10,65,66]. The re-definition of treatment goals[67-70] and the revision of current treatment strategies[71-75] promise to improve results. The emergence of powerful immunosuppressive agents, mainly from the transplantation arena, promises to strengthen the treatment repertoire[76,77], and the clarification of critical pathogenic pathways make site-specific molecular interventions feasible[76,78,79]. The clinical spectrum of autoimmune hepatitis has expanded, but the diagnostic instruments and therapeutic options have also improved.

The objectives of this review are to describe the non-classical clinical phenotypes of autoimmune hepatitis, detail the diagnostic instruments that can ensure their recognition, and introduce the evolving treatment strategies. Classical syndromes are the cornerstones of clinical practice, but variations from the classical are its realities. The changing spectrum of autoimmune hepatitis and its treatment underscores the importance of the disease and the vigor of the investigative effort that it has generated.

NON-CLASSICAL CLINICAL PHENOTYPES

Autoimmune hepatitis is defined as a self-perpetuating inflammation of the liver of unknown cause that is characterized by interface hepatitis on histological examination, hypergammaglobulinemia, and autoantibodies[80]. The diagnosis has great latitude since no features are disease-specific. The phenotypes that satisfy the definition of autoimmune hepatitis but are outside the boundaries of classical disease have acute severe presentations, few or no symptoms, atypical histological findings, absent or variant serological markers, concurrent cholangiographic changes, male gender, and non-Caucasian backgrounds.

Table 1 Non-classical phenotypes of autoimmune hepatitis

Non-classical phenotype	Salient features
Acute severe disease	Corticosteroids effective in 36%-100%[49] Protracted treatment can be complicated by infection[49] High mortality if no better within 2 wk of therapy[85] MELD score ⩾ 12 identifies 97% of treatment failures[58]
Asymptomatic mild hepatitis	Common (25%-34%) but unstable state[20-22,87-89] Symptoms develop in 26%-70%[20,21] Progression possible if untreated[20-22,87] Improves quickly with therapy[22]
Atypical histological features	Centrilobular necrosis is an early acute form[18,33-37,92] Transition to interface hepatitis possible[35] Coincidental biliary changes lack cholestatic profile[41] Fatty changes may co-exist[58,94]
Absent or variant serological markers	Seronegativity possible in 13%[31] Other features and treatment outcome similar[31,32,100] Non-standard autoantibodies possible[101-104] Conventional autoantibodies may be expressed later[30] Screen for celiac disease[105-108]
Concurrent cholangiographic changes	Abnormal cholangiograms in 44% with CUC[116] Poor outcome if biliary changes and CUC[121-124] MRC abnormalities in 8% adults without CUC[117] MRC abnormalities may be associated with fibrosis[119]
Male gender	0.2-0.5 cases/100 000 per year[127,128] Low frequency of concurrent immune diseases[130-132] No diversity of HLA DRB1*04 alleles[130-132] Better survival than women[136]
Non-Caucasian	Cholestatic features may be common[45,141-143] Male predominance possible[47] Socioeconomic factors important[137,138,146,147]

MELD: Model of End Stage Liver Disease; MRC: Magnetic resonance cholangiography; CUC: Chronic ulcerative colitis; HLA: Human leukocyte antigen.

Acute severe presentations

The diagnosis of autoimmune hepatitis is no longer restricted by a time requirement for disease activity[9,10]. An acute severe or fulminant presentation can reflect *de novo* inflammation[12-15] or the spontaneous exacerbation of a previously unsuspected chronic disease[81]. An acute or abrupt onset occurs in 40% of patients with autoimmune hepatitis[14,81,82], whereas an acute severe presentation is rare[83] (Table 1).

The acute form can be differentiated from the chronic form by laboratory features [higher serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels, total serum bilirubin concentration, and serum γ-glutamyl transpeptidase level] and by histological findings (more frequent centrilobular zone 3 necrosis with plasmacytic infiltration and bile duct injury), but its recognition in individual cases relies mainly on an awareness that acute severe autoimmune hepatitis is possible[84].

Corticosteroid therapy suppresses inflammatory

activity in 36%-100% of patients with acute severe presentations[49], whereas delay in treatment can result in a poor outcome[51,83] (Table 1). Immediate survival and the need for urgent transplantation depend on the rapidity and nature of the response to corticosteroids[50,85,86]. Failure to improve at least one laboratory abnormality reflective of liver inflammation or function, especially a pre-treatment hyperbilirubinemia, within 2 wk indicates that liver transplantation should be considered[85]. Relentless pursuit of an unachievable benefit from corticosteroid therapy can be complicated by infection and the lost opportunity for a successful transplantation[49].

The Model of End Stage Liver Disease (MELD) can be used to identify individuals with autoimmune hepatitis who are likely to fail corticosteroid therapy and require liver transplantation[58] (Table 1). MELD scores $\geqslant$ 12 points at presentation have a sensitivity of 97% and specificity of 68% for treatment failure, and patients with such scores warrant close scrutiny and preparedness for liver transplantation. A MELD score $\geqslant$ 12 points at presentation captures all problematic patients, but it does not preclude their salvation through prompt and vigorous corticosteroid treatment.

Asymptomatic mild presentations

Autoimmune hepatitis is asymptomatic in 25%-34% of patients at presentation[20-22], and retrospective analyses have estimated that 25%-85% of individuals have mild disease[20-22,87-89] (Table 1). These presentations contrast with those described in the classical treatment trials in which selection criteria focused on severe symptomatic and immediately life-threatening disease[51,90,91]. Treatment guidelines have been promulgated for the individuals with severe disease, but they remain arbitrary and inconsistent for those with mild disease[80]. These difficulties reflect in part the lack of a codified definition of mild autoimmune hepatitis and uncertainty about its natural history.

Untreated mild autoimmune hepatitis has a better outcome than severe disease, but it does not have a benign prognosis (Table 1). Cirrhosis develops in 49% of untreated patients within 15 years[87]; liver failure and hepatocellular carcinoma are possible[22]; asymptomatic patients become symptomatic in 26%-70% of instances[20,21]; and 10-year mortality exceeds 10%[21,22]. Spontaneous resolution is possible, but untreated patients with mild autoimmune hepatitis improve less commonly (12% *vs* 63%, $P = 0.006$) and more slowly than treated patients, and they have a lower 10-year survival (67% *vs* 98%, $P = 0.01$)[22].

A "safe" subset of patients with non-aggressive autoimmune hepatitis who require no therapy cannot be reliably identified, and the clinical threshold for starting corticosteroid therapy cannot be so high that all patients with mild or asymptomatic disease are excluded (Table 1). Mild autoimmune hepatitis can improve spontaneously, and this prospect may dampen therapeutic zeal, especially if measured against the possibility of serious treatment-related complications[22]. A dictum to do no harm, however, that focuses more concern on the treatment than the disease may be incorrect.

The aggressive potential of mild autoimmune hepatitis at presentation, the inability to predict outcome by clinical parameters, the expected rapidity of the treatment response, and the safety of current treatment regimens favor a proactive management strategy[22]. Until randomized clinical trials are performed comparing treatment against no treatment, the management strategy in patients with mild disease should lean toward conventional therapy. Mild asymptomatic autoimmune hepatitis is a non-classical phenotype, but it should not be regarded or managed as a different disease.

Atypical histological features

The histological hallmark of autoimmune hepatitis is interface hepatitis, but other histological findings are compatible with the disease[9,10] (Table 1). Centrilobular zone 3 necrosis (Figure 1) is probably an early form of autoimmune hepatitis that is detected mainly in patients with an acute onset[18,33-37,92]. Successive liver tissue examinations have disclosed transition of the centrilobular zone 3 pattern of necrosis to that of typical interface hepatitis during the course of the disease[35]. This non-classical finding may suggest an acute viral or toxic injury, but the diagnosis of autoimmune hepatitis should not be discounted.

Concurrent biliary changes, including isolated destructive cholangitis (Figure 2), may also be found in patients with otherwise classical autoimmune hepatitis[38-42] (Table 1). These patients do not have a cholestatic clinical or laboratory profile, and successive tissue examinations have not disclosed persistence or progression of the biliary injury[41]. The biliary changes probably reflect collateral damage associated with an exuberant inflammatory process rather than a transition state to a cholestatic disease or variant syndrome. The biliary changes should not alter the diagnosis or the treatment strategy.

Fatty changes (Figure 3) may also be present at accession or after corticosteroid therapy[58,93,94] (Table 1). Non-alcoholic fatty liver disease (NAFLD) is a common finding in the general population, and it may be associated with autoantibodies and hypergammaglobulinemia[94-97]. Both conditions can co-exist, and corticosteroid therapy can ameliorate the autoimmune hepatitis and intensify the NAFLD[58,94]. The presence of coincidental fatty change should not discourage the diagnosis or treatment of autoimmune hepatitis, but it compels an accurate diagnosis. Worsening of the laboratory indices during therapy justifies liver tissue examination and reassessment of the treatment strategy[58,94]. Progressive fatty change can be a cause of treatment failure[58].

Absent or atypical serological markers

Antinuclear antibodies (ANA), smooth muscle antibodies (SMA), and antibodies to liver kidney microsome type 1 (anti-LKM1) are the classical serological markers of autoimmune hepatitis[98,99]. These antibodies are not pathogenic or disease-specific, and their expression can vary in individual cases and in different geographical

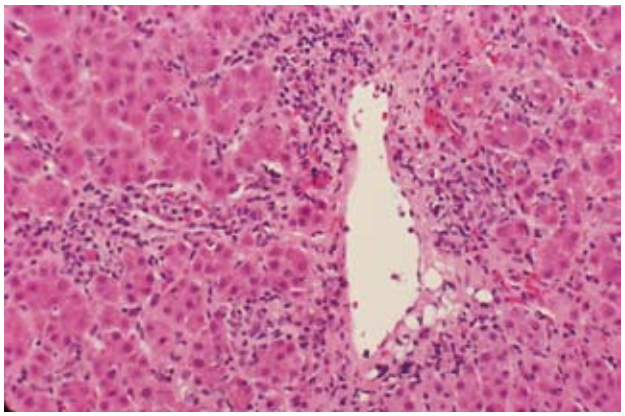

Figure 1 Centrilobular zone 3 necrosis. Inflammation and hepatocyte drop out are present around a terminal hepatic venule in conjunction with hepatic plate thickening, architectural disorganization, and rosette formation. Centrilobular (perivenular) zone 3 necrosis can be an early acute form of autoimmune hepatitis that can transform to interface hepatitis (HE, × 200).

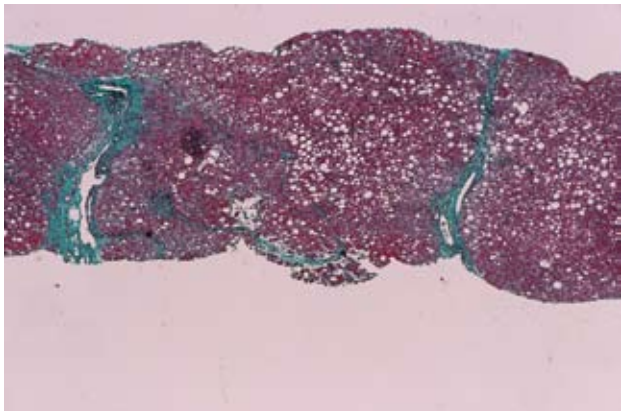

Figure 3 Steatosis. Macrovesicular steatosis is the predominant histological feature after corticosteroid treatment. Fatty changes may be present before or during corticosteroid treatment and perpetuate or extend the laboratory indices of liver inflammation (Trichrome stain, × 40).

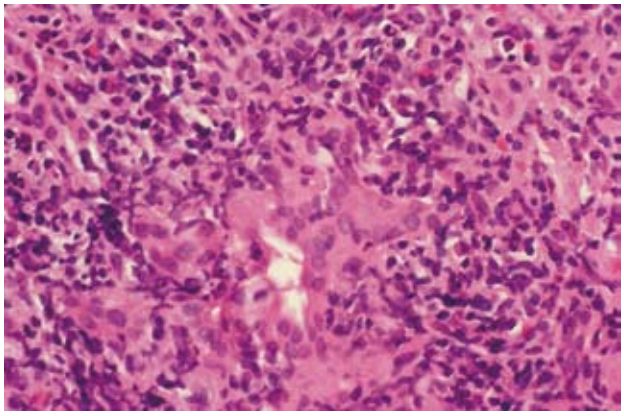

Figure 2 Concurrent pleomorphic cholangitis. Lymphocytes and histiocytes surround, infiltrate and damage an interlobular bile duct. Bile duct injury in the absence of cholestatic clinical and laboratory manifestations may represent collateral injury that is transient (HE, × 400).

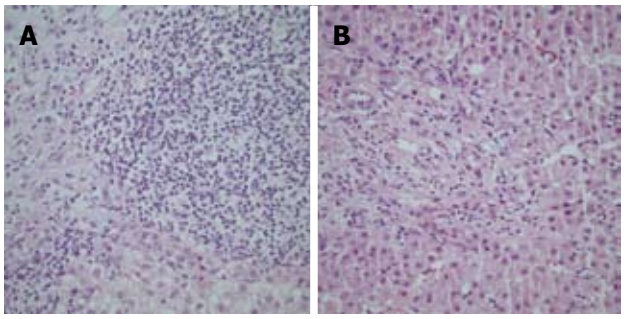

Figure 4 Histological features of a Turkish patient with the "overlap syndrome" (autoimmune hepatitis and primary biliary cirrhosis) characterized by heavy portal infiltration with lymphocytes and plasma cells (A) and bile ductular proliferation and ductopenia (B) (HE, × 200).

regions and ethnic groups. Thirteen percent of white North American adults with classical features of autoimmune hepatitis lack ANA, SMA, and anti-LKM1[31] (Table 1).

Seronegative patients with autoimmune hepatitis have a non-classical phenotype, and they constitute an "autoantibody-negative autoimmune hepatitis"[31,32,100]. These patients are indistinguishable from those with classical disease, including their HLA profiles, and they also respond to corticosteroid therapy[31,32,100] (Table 1). Twenty percent may express non-standard autoantibodies, such as antibodies to soluble liver antigen (anti-SLA)[101,102] or atypical perinuclear anti-neutrophil cytoplasmic antibodies (atypical pANCA)[103,104], and others may express SMA, ANA or both later in their course[30]. Some corticosteroid-responsive patients remain seronegative throughout their disease, and they may await discovery of their signature autoantibody[31,32,100]. All such patients must be screened for celiac disease by testing for immunoglobulin A antibodies to tissue transglutaminase or endomysium[105-108].

Antimitochondrial antibodies (AMA) can be present in 8%-35% of patients with otherwise classical autoimmune hepatitis, and they define another non-classical serological phenotype[108-112]. These coincidental AMA are not associated with cholestatic features, histological findings of biliary injury, or different response to corticosteroid therapy[110-113]. They may persist for as long as 27 years in the absence of primary biliary cirrhosis (PBC)[111]; they may disappear spontaneously; or they may appear late in the course of the disease without apparent clinical relevance[112]. Severe inflammatory activity may result in modification of the mitochondrial antigens through oxidative stress and facilitate the production of AMA which in turn can disappear when the inflammatory stress subsides[114]. AMA in the absence of cholestatic laboratory or histological features should not dissuade the diagnosis of autoimmune hepatitis or compel a different treatment strategy. The "serological overlap" with PBC does not constitute a hybrid disease or pathological process in transition.

Concurrent cholangiographic changes

Concurrent cholangiographic changes have been described in children[115] and adults[116,117] with autoimmune hepatitis, and these findings constitute another non-classical clinical phenotype (Table 1). The

emergence of magnetic resonance cholangiography (MRC) as a safe, effective and non-invasive mechanism by which to assess the biliary system[118] has indicated that cholangiographic changes that resemble primary sclerosing cholangitis (PSC) occur in 8% of adults with autoimmune hepatitis[117]. These changes occur predominately in women who typically lack inflammatory bowel disease, and they are associated with histological features that reflect increased lobular activity rather than biliary injury[117].

The nature and significance of the biliary changes by MRC remain unclear since most adults with these changes respond to corticosteroid therapy[117]. The possibility of a disease process other than typical PSC or an unusual but nonspecific biliary distortion induced by fibrosis cannot be discounted (Table 1). Recent prospective studies have indicated that while adults with autoimmune hepatitis have a high frequency of intrahepatic biliary changes by MRC (24%), the occurrence of PSC is rare (2%)[119]. Furthermore, the frequency of biliary changes by MRC in adults with autoimmune hepatitis is similar to that in patients with cirrhosis of a non-biliary and non-autoimmune nature. Hepatic fibrosis rather than the nature of the liver disease may be the most important parameter independently associated with the biliary changes[119].

Cholangiographic abnormalities by endoscopic or intrahepatic cholangiography are present in 44% of adults with autoimmune hepatitis and inflammatory bowel disease[116,120], and patients with these changes are typically refractory to corticosteroid therapy[121-124] (Table 1). This is the non-classical phenotype that has immediate clinical relevance, and its discovery impacts on the diagnosis, treatment, and outcome. Biliary studies should be performed mainly in adult patients with inflammatory bowel disease or recalcitrance to corticosteroid therapy[121,125]. Not all biliary changes have independent pathological significance or clinical importance[126].

Male gender

Autoimmune hepatitis does occur in white northern European men[127,128], and its development in this gender constitutes another non-classical phenotype (Table 1). Women with autoimmune hepatitis outnumber men with the disease by more than three-fold[129], and estimates of the incidence of autoimmune hepatitis in northern European men ranges from 0.2-0.5 cases per 100 000 persons per year[127,128]. The existence of an important clinical distinction between men and women with autoimmune hepatitis is still unsettled, but clearly the experiences over the decades have not identified a striking difference between the genders.

White North American women with autoimmune hepatitis are distinguished from men with autoimmune hepatitis and the same ethnicity by having higher frequencies of concurrent immune diseases (34% *vs* 17%, $P = 0.05$) and HLA DRB1*04 (49% *vs* 24%, $P = 0.007$)[130-132] (Table 1). Women with HLA DRB1*04 also have higher frequencies of concurrent immune diseases than women without HLA DRB1*04 (52% *vs* 22%, $P < 0.000001$) as do men with HLA DRB1*04 compared to men without HLA DRB1*04 (26% *vs* 4%, $P = 0.002$)[129,132]. These findings suggest that the clinical phenotype is driven by the genetic predisposition of the host as well as the gender[129,133].

Retrospective surveys have suggested gender-based differences in disease behavior and treatment outcome, but results have been discrepant[134-136]. Differences in age at presentation (39 years *vs* 49 years, $P = 0.06$) and the frequency of relapse after drug withdrawal (71% *vs* 55%, $P = 0.06$) have not reliably distinguished men from women with autoimmune hepatitis. The higher frequency of HLA A1-B8-DRB1*03 in men who relapse (50% *vs* 23%, $P = 0.003$) and greater mortality in women than men ($P = 0.02$) have been contrasting features in some experiences, and these findings require further examination[135,136] (Table 1).

The principal clinical concern related to gender is that the diagnosis of autoimmune hepatitis might be overlooked in men. Gender may be a surrogate marker that signifies different antigenic exposures, hormonal effects on immune responsiveness, chromosomal imbalances that favor loss of self-tolerance, and genetic predispositions for immunocyte activation[132]. The diagnosis of autoimmune hepatitis in men should trigger the same treatment strategies and monitoring schedules as in women.

Non-Caucasians

Racial background can affect the clinical phenotype of autoimmune hepatitis, and diagnostic criteria developed mainly in white northern European and North American populations may not apply in different ethnic groups and geographical regions (Table 1). Black North American patients have cirrhosis at presentation more commonly than white North American patients (85% *vs* 38%)[43,137,138]. Japanese patients typically have mild, late onset disease[139]. South American patients are younger than white North American counterparts, and they have more severe laboratory abnormalities at presentation[140]. Alaskan natives have a higher frequency of acute icteric disease, asymptomatic illness, and advanced fibrosis at accession than non-native patients[44]. Aboriginal North Americans have disproportionately high frequencies of immune-mediated disorders, cholestatic features, and advanced disease at presentation[141-143]. African, Asian and Arab patients have a higher frequency of biliary changes on histological examination than white northern European patients[45], and patients from Somalia are frequently men with rapidly progressive disease[47] (Table 1).

The variations in clinical phenotype suggest that genetic background and geographical location affect occurrence and behavior of the disease[129,133]. Indigenous etiological agents or population-dependent genetic factors may modulate susceptibility to autoimmune hepatitis, determine targets of the immune response, and affect the vigor of the inflammatory reaction[144,145]. Socioeconomic status, healthcare access, and quality of care are other factors that must be analyzed when assessing discrepancies

in disease occurrence and outcome among different racial groups[137,138,146,147] (Table 1).

TURKISH PERSPECTIVE

The importance of recognizing the diverse manifestations of autoimmune hepatitis in different regions and ethnic groups is illustrated by the appearance and behavior of the disease in Turkey. Autoimmune hepatitis in this region has a character that aligns with the disease of white northern Europeans and North Americans, but it can be difficult to recognize if only the western phenotype is considered.

Autoimmune hepatitis is a relatively rare disorder in Turkey when compared with chronic viral hepatitis, but it is still the most common autoimmune liver disease[148,149]. Its high prevalence in women is not unusual, but its 9:1 female-to-male ratio[148,149] exceeds the female propensity (3:1 female-to-male ratio) in North America[129]. The age of occurrence in adults (age ranges, 18-59 years; mean age, 42 years) is as broad as elsewhere[150], but there are many patients with signs of hepatitis who are negative for viral markers and the conventional autoantibodies[149]. These patients are typically designated as having "cryptogenic chronic hepatitis", but they respond well to treatment with corticosteroids and azathioprine. Other liver diseases must be carefully excluded, especially in men and those who lack the conventional autoantibodies, and the diagnosis must be supported by the demonstration of compatible liver enzyme abnormalities, serum immunoglobulin G (IgG) elevation, and histological findings. The presence of periportal lymphoplasmacytic infiltration in liver tissue is an important clue to the diagnosis, and all patients in whom there is a suspicion of autoimmune hepatitis should undergo liver tissue examination.

As in other regions, the features of autoimmune hepatitis may be intermixed with those typical of other liver diseases, especially the cholestatic disorders ("overlap syndromes"), and the diagnosis must be secured by expert histological interpretation and cholangiographic studies (Figure 4)[148,151,152]. In Turkey, as elsewhere, liver tissue examination is the most important tool in directing the diagnosis, and a second examination of the liver tissue after institution of treatment provides a comparison that can support or change the original impression.

Concurrent immune diseases, such as autoimmune thyroiditis[153], celiac disease[154], Sjogren syndrome[155], autoimmune diabetes[155], and various rheumatic conditions[156], can accompany the autoimmune hepatitis of Turkey, but unlike the disease in other regions, the liver disease in Turkey can frequently be linked to different triggers, including indigenous infections [prolonged hepatitis A virus (HAV) infection[157] and brucellosis[158]] and medicinal agents (Echinacea[159], doxycycline[158], estrogen[160], cyproterone acetate[160], and ornidazole[161]). A genetic basis for the liver disease and its immune manifestations has not been well studied in Turkey, but the classical HLA phenotype, A1-B8-DRB1*03, of western countries does not appear to be an important susceptibility factor in this area[162].

Corticosteroid therapy remains the mainstay of treatment in Turkey, but azathioprine, ursodeoxycholic acid and budesonide have been added to the list of available and effective drugs. Combination therapy is the preferred regimen, and budesonide is gaining favor over prednisolone in combination with azathioprine. An example of tailoring the treatment strategy to the population base is the practice of maintaining individuals in remission on low dose prednisolone (4 mg on alternate days) either alone or in combination with azathioprine long-term. By recognizing the regional variations in the clinical phenotype and tailoring therapy to suit the prevalent disease behavior, autoimmune hepatitis in different regions and ethnic groups can be diagnosed promptly and treated successfully in a cost-effective, low risk manner.

NEW DIAGNOSTIC INSTRUMENTS

New diagnostic instruments have evolved that have the flexibility to accommodate atypical features of autoimmune hepatitis and the sensitivity and specificity to ensure accurate diagnosis of the non-classical phenotypes. A diagnostic scoring system that was promulgated mainly as a research tool in 1993[9] was revised in 1997[10] to exclude cholestatic syndromes. A simplified diagnostic scoring system was added in 2008 to ease clinical application[65], and both systems can now be exploited to strengthen the diagnosis in difficult cases[66].

Original revised diagnostic scoring system

The revised original diagnostic scoring system developed by the International Autoimmune Hepatitis Group (IAIHG) evaluates 13 clinical components and renders 27 possible grades[10] (Table 2). It is a comprehensive template that grades each component of the disease, including gender, laboratory manifestations of liver inflammation and cholestasis, the conventional autoantibodies, viral markers, epidemiological risk factors such as drug or alcohol exposures, HLA phenotype, concurrent immune diseases, novel autoantibodies, and individual histological features. It also grades the treatment response, and a score can be rendered before and after treatment.

A pre-treatment score of 10 points or higher or a post-treatment score of 12 points or higher indicates the likelihood of autoimmune hepatitis[10] (Table 2). No single test or finding defeats or ensures the diagnosis of autoimmune hepatitis if other components are sufficiently strong to outweigh it. The Receiver Operating Characteristic (ROC) curve for the revised original scoring system shows that the minimum pre-treatment score of 10 points has a sensitivity of 100% and a specificity of 73% for autoimmune hepatitis. A pre-treatment score of 15 points or higher has a specificity of greater than 90% for autoimmune hepatitis[66].

The principal virtues of the revised original scoring

Table 2 Revised original pre-treatment scoring system[10]

Variable	Result	Points	Variable	Result	Points
Gender	Female	+2	HLA	DR3 or DR4	+1
AP:AST (or ALT) ratio	> 3	-2	Immune disease	Thyroiditis, colitis, others	+2
	< 1.5	+2			
γ-globulin or IgG level above normal	> 2.0	+3	Other markers	Anti-SLA, actin, LC1, pANCA	+2
	1.5-2.0	+2			
	1.0-1.5	+1			
	< 1.0	0			
ANA, SMA, or anti-LKM1 titers	> 1:80	+3	Histological features	Interface hepatitis	+3
	1:80	+2		Plasmacytic	+1
	1:40	+1		Rosettes	+1
	< 1:40	0		None of above	-5
				Biliary changes	-3
				Other features	-3
AMA	Positive	-4	Treatment response	Complete	+2
				Relapse	+3
Viral markers	Positive	-3			
	Negative	+3			
Drugs	Yes	-4	Pretreatment aggregate score		
	No	+1	Definite diagnosis		> 15
			Probable diagnosis		10-15
Alcohol	< 25 g/d	+2	Post-treatment aggregate score		
	> 60 g/d	-2	Definite diagnosis		> 17
			Probable diagnosis		12-17

AP: AST (or ALT) ratio: Ratio of alkaline phosphatase level to aspartate or alanine aminotransferase level; Anti-SLA: Antibodies to soluble liver antigen; Anti-LC1: Antibodies to liver cytosol type 1; pANCA: Perinuclear anti-neutrophil cytoplasmic antibodies; IgG: Immunoglobulin G; ANA: Antinuclear antibodies; SMA: Smooth muscle antibodies; Anti-LKM1: Antibodies to liver/kidney type 1; AMA: Antimitochondrial antibodies; HLA: Human leukocyte antigen.

Table 3 Simplified scoring system of the International Autoimmune Hepatitis Group[65]

Variable	Result	Points
Autoantibodies		
ANA or SMA	≥ 1:40	+1
ANA or SMA	≥ 1:80	+2
Antibodies to liver kidney microsome type 1	≥ 1:40	+2
Antibodies to soluble liver antigen	Positive	+2
Immunoglobulin level		
Immunoglobulin G	> UNL	+1
	> 1.1 ULN	+2
Histological findings		
Morphological features	Compatible	+1
	Typical	+2
Viral disease		
Absence of viral hepatitis	No viral markers	+2
Pretreatment aggregate score		
Definite diagnosis		≥ 7
Probable diagnosis		6

ULN: Upper limit of normal.

system are that it ensures the systematic assessment of all key features of the disease and it is not compromised by a missing or atypical feature[66]. The revised original diagnostic scoring system is most useful in evaluating patients with few or atypical findings of autoimmune hepatitis, including the variant syndromes, because of its comprehensive nature. It quantifies the strength of the diagnosis, and it is a valuable research tool that ensures comparable study populations within clinical trials.

Simplified diagnostic scoring system

The simplified diagnostic scoring system eases clinical application by evaluating only 4 clinical components, and it has been validated in diverse ethnic groups and liver diseases[65] (Table 3). The simplified scoring system is based on the presence and level of autoantibody expression by indirect immunofluorescence, serum IgG concentration, compatible or typical histological features, and the absence of viral markers. It does not grade treatment response.

The ROC curve for the simplified scoring system shows that a minimum score of 6 points has a sensitivity and specificity of 90% for the diagnosis of autoimmune hepatitis[66] (Table 3). Scores of 7 points or higher are nearly 100% specific for the diagnosis of autoimmune hepatitis with only a small decrease in sensitivity. The virtues of the simplified scoring system are the ease of its clinical application, and its combined high sensitivity and specificity for the diagnosis[66]. It is especially useful in excluding autoimmune hepatitis in patients with other distinct conditions who have confusing concurrent immune features. The revised original scoring system has greater sensitivity for the diagnosis, whereas the simplified system has superior specificity and accuracy.

NEW TREATMENT STRATEGIES

New treatment strategies for autoimmune hepatitis are evolving as current regimens are being used more effectively and new drugs are being exploited in selected situations. The non-classical phenotypes of autoimmune hepatitis are managed in the same fashion as the classical phenotypes, and they benefit similarly from these advances.

Table 4 Therapeutic advances in autoimmune hepatitis

Advance	Nature	Attribute
Improved current therapy	Initial therapy until resolution of liver tests and tissue	Prevention of relapse after drug withdrawal[70]
	Long-term azathioprine therapy after relapse or incomplete response	Prevention of disease progression[71,73,125]
	Pretreatment vaccination for viruses	Protection against morbidity of concurrent viral infection[74]
New drugs	Calcineurin inhibitors (cyclosporine, tacrolimus)	Salvage therapy[150-157]
	Purine antagonists (6-mercaptopurine, mycophenolate)	Salvage therapy[161-166]
	Budesonide (combined with azathioprine)	Effective and safe front line therapy[75]
Potential molecular interventions	Synthetic blocking peptides	Block autoantigen presentation[186,187]
	Cytokine manipulations	Promote anti-inflammatory effects[188]
	T cell vaccination	Eliminate cytotoxic liver-infiltrating clone[189]
	Oral tolerance (high or low dose regimen)	Reduce immune response (low dose) or induce anergy (high dose)[190,191]
	Mesenchymal stem cells (human bone marrow-derived)	Replace damaged hepatocytes[200]

Improvements in current treatment strategies

The ideal end point of initial corticosteroid therapy has now been defined[67-70]; the treatment adjustments after relapse and incomplete response have been formalized[71-73]; and vaccination against hepatitis A (HAV) and hepatitis B (HBV) viruses prior to therapy has been proposed[74]. These improvements constitute advances in the current treatment regimens (Table 4).

Corticosteroid therapy should be continued until the clinical, laboratory and histological features of autoimmune hepatitis have fully resolved (Table 4)[67-70]. Relapse after drug withdrawal is the most common management problem in autoimmune hepatitis, and this occurrence can be reduced by continuing treatment until liver tests and tissue are normal[59,70,163]. Patients who sustain remission after treatment withdrawal have better laboratory indices and liver tissue examinations at the time of drug withdrawal than patients who relapse, and treatment until complete resolution of the inflammatory features is the ideal end point of therapy.

Sixty percent of patients who achieve an ideal treatment end point still relapse after drug withdrawal, and 40% of treated patients are unable to achieve full resolution of their disease[70]. The relentless pursuit of an ideal but unachievable treatment end point in these individuals can result in drug-related side effects[59,62]. Patients with relapse after drug withdrawal, incomplete response to conventional treatment, and drug intolerances must be managed differently[125].

Repeated relapse and re-treatment is associated with a progressive increase in the cumulative frequencies of cirrhosis, requirement for liver transplantation, and death from hepatic failure[64]. The preferred management strategy after the first relapse is to institute treatment with long-term fixed dose azathioprine (Table 4)[71,73]. Prednisone and azathioprine are re-started until clinical and laboratory resolution is achieved. The dose of azathioprine is then increased to 2 mg/kg daily as the dose of prednisone is withdrawn. Azathioprine is then continued indefinitely as a chronic maintenance therapy. Eighty percent of patients are able to sustain remission in this fashion over a 10 year period of observation. Patients who improve during treatment but not to a degree to satisfy remission criteria (incomplete response) can also be managed by this regimen[125].

Vaccination against HAV and HBV is an important adjunct to conventional treatment (Table 4). Susceptibility to infections with HAV (51%) and HBV (86%) is high in patients with autoimmune liver disease, and the incidence of these infections is 1.3-1.4 per 1000 person-years[74]. Vaccination frequencies are only 11% for HBV and 13% for HAV in these patients, and the response to the HBV vaccine is poor or absent in most individuals vaccinated during immunosuppressive therapy[74]. These observations suggest that pre-treatment vaccination for HAV and HBV is under-utilized in autoimmune liver disease and that outcomes can be improved by early vaccination to prevent viral super-infection and mortality.

Advances in pharmacological options

Treatment options have increased in autoimmune hepatitis as new drugs with targeted immunosuppressive actions have been used empirically[76,164] and a third generation corticosteroid has been evaluated by randomized clinical trial[75]. None of these treatments has been incorporated into standard management algorithms, but they constitute an evolving armamentarium that promises to improve outcomes by either interrupting critical pathogenic pathways or eliminating intolerances to the current medications.

The calcineurin inhibitors (cyclosporine[165-169] and tacrolimus[170-172]) have been used as frontline and salvage therapies in children[173-175] and adults[165-172] with autoimmune hepatitis, and these multiple small clinical experiences have supported their efficacy and tolerance (Table 4). Additional clinical trials are necessary to determine their target population, dosing schedule, and safety profile.

The purine antagonists (6-mercaptopurine[176] and mycophenolate mofetil[177-181]) have also been effective in some patients refractory to conventional corticosteroid regimens (Table 4). 6-mercaptopurine has reduced disease activity in patients unresponsive to azathioprine, and should be considered as a salvage therapy[176]. Intolerances to azathioprine based on thiopurine methyltransferase deficiency contraindicate its use, and the drug should not be administered to patients with

azathioprine-related side effects[182].

Mycophenolate mofetil is independent of the thiopurine methyltransferase metabolic pathway, and several small experiences have indicated that it can be effective in problematic patients[177-181] (Table 4). Improvement occurs in 39%-84% of patients who tolerate the drug, but the intention to treat is thwarted in 34%-78% of patients because of drug intolerances (nausea, vomiting, pancreatitis, rash, alopecia, deep venous thrombosis, diarrhea and failure to normalize liver tests)[180,183,184]. Mycophenolate mofetil is another promising alternative drug in the treatment of autoimmune hepatitis, but only a minority of problematic patients may reap its benefits[183-185].

Budesonide is a third generation corticosteroid that has been used empirically as frontline[186-188] and salvage[189] therapy in autoimmune hepatitis (Table 4). Its high first-pass clearance by the liver and its breakdown to inactive metabolites promised to improve efficacy and safety compared to conventional corticosteroid regimens[190]. Its advantage, however, was never fully realized until it was evaluated by randomized clinical trial in 203 treatment-naïve patients with autoimmune hepatitis[75]. Budesonide in combination with azathioprine has been found to be superior to prednisolone and azathioprine in normalizing the serum ALT level (47% *vs* 18%, $P < 0.00001$) and reducing the frequency of steroid-related side effects (28% *vs* 53%, $P = 0.0001$) after 6 mo of treatment[75]. The frequency of histological improvement and the durability of the results are unknown, but the findings suggest that budesonide may be an alternative, more effective, and safer frontline regimen than a prednisone-based schedule. Budesonide has not been effective as a salvage therapy in patients with severe disease on long-standing corticosteroid treatment[189], and corticosteroid-induced side effects are still possible, especially in patients who have been treated previously with prednisone[189] or who have cirrhosis[189,191].

Various other drugs (cyclophosphamide[192], methotrexate[193], rapamycin[194], rituximab[195], intravenous immunoglobulin[196], deflazacort[197], and ursodeoxycholic acid[198]) have been proposed for use in autoimmune hepatitis, and their number reflects the need for better salvage therapies in the treatment of autoimmune hepatitis (Table 4). Prospective and scientifically rigorous collaborative studies are needed to expand the therapeutic repertoire and comprehensive analyses are required to demonstrate that these incremental improvements in outcome are cost-effective[199,200].

Site-specific molecular inventions, including antigen-blocking synthetic peptides[201,202], cytokine manipulations[203], T cell vaccination[204], and oral tolerance regimens[205,206], become feasible when the critical pathogenic mechanisms of the disease are clarified[207-209], and confident animal models of the human disease are developed[210-214] (Table 4). Mesenchymal stem cells from human bone marrow that can differentiate into functional hepatocytes have the potential to rescue individuals from liver failure, reduce reliance on whole organ transplantation, and obviate the complications of whole organ rejection and drug toxicity[215] (Table 4). The treatment options for autoimmune hepatitis are already plentiful and effective, but the drive for further improvement must be continuous and vigorous.

CONCLUSION

Autoimmune hepatitis can have acute severe or asymptomatic presentations, centrilobular zone 3 necrosis, concurrent bile duct damage or non-alcoholic fatty changes on histological examination, absent or atypical serological markers, cholangiographic abnormalities, and variable clinical phenotypes related to gender or ethnicity. These non-classical manifestations do not alter the management strategy, but they require prompt recognition and confident diagnosis. The revised original diagnostic scoring system of the IAIHG is useful in evaluating patients with few or atypical findings of autoimmune hepatitis because of its comprehensive nature. The simplified diagnostic scoring system is useful in excluding autoimmune hepatitis in patients with other distinct conditions who have confusing concurrent immune features because of its high specificity. Current treatment regimens have been improved by pursuing resolution of liver tests and liver tissue prior to drug withdrawal, instituting long-term azathioprine therapy after the first relapse, and vaccinating against HAV and HBV prior to treatment. Cyclosporine, tacrolimus, 6-mercaptopurine, and mycophenolate mofetil are promising salvage therapies, whereas budesonide in combination with azathioprine may be a superior frontline therapy to prednisone and azathioprine.

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Signal transduction mechanism of TRB3 in rats with non-alcoholic fatty liver disease

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Abstract

AIM: To evaluate the possible role of Tribble 3 (TRB3) in a rat model of non-alcoholic fatty liver disease (NAFLD) and its signal transduction mechanism.

METHODS: Thirty Sprague-Dawley rats were randomized into three groups: normal control group, non-alcoholic fatty liver group A (fed on a high-fat diet for 8 wk) and group B (fed on a high-fat diet for 16 wk). To determine the degree of hepatic steatosis in rats of each group, livers were stained with hematoxylin and eosin, and evaluated; real-time fluorescent quantitative reverse transcriptase-polymerase chain reaction was performed to measure the expression levels of TRB3 mRNA; and Western blotting analysis was done to determine the expression levels of protein kinase B (Akt) and phosphorylated protein kinase B (p-Akt-Thr308, p-Akt-Ser473).

RESULTS: Hepatic steatosis was evident in both NAFLD groups: mild to moderate hepatic steatosis occurred in group A, mainly as mild steatosis. Moderate to severe hepatic steatosis occurred in group B, mainly as severe steatosis. The expression level of TRB3 mRNA in group B was significantly higher than in the control group (122.28 ± 95.37 *vs* 3.06 ± 2.33, $P = 0.001$) and group A (122.28 ± 95.37 *vs* 5.77 ± 4.20, $P = 0.001$). There was no significant difference in the expression levels of Akt (1.03 ± 0.53 *vs* 1.12 ± 0.77, $P = 0.729$) and p-Akt-Thr308 (0.82 ± 0.45 *vs* 0.92 ± 0.38, $P = 0.592$) between group A and the control group. The expression level of Akt and p-Akt-Thr308 in group B was significantly lower than in group A (Akt 0.41 ± 0.16 *vs* 1.12 ± 0.77, $P = 0.008$; p-Akt-Thr308 0.47 ± 0.19 *vs* 0.82 ± 0.45, $P = 0.036$) and the control group (Akt 0.41 ± 0.16 *vs* 1.03 ± 0.53, $P = 0.018$; p-Akt-Thr308 0.47 ± 0.19 *vs* 0.92 ± 0.38, $P = 0.010$). The expression level of p-Akt-Ser473 in group A was significantly higher than in group B (1.48 ± 0.50 *vs* 0.81 ± 0.39, $P = 0.041$) as well as the control group (1.48 ± 0.50 *vs* 0.45 ± 0.26, $P = 0.003$).

CONCLUSION: TRB3 blocks insulin signaling by inhibiting Akt activation, which contributes to insulin resistance. It may be an important factor in the occurrence and development of NAFLD.

Key words: Non-alcoholic fatty liver disease; Rat; Tribble 3; Protein Kinase B; Insulin resistance

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INTRODUCTION

Three pseudo kinases of the Tribble family[1,2] have been recognized recently, which include Tribble 1 (TRB1), TRB2 and TRB3. Different from the typical kinase domain structure, tribbles lack a conventional ATP binding site and activity domain of protein kinases. Thus, no kinase activity by tribbles has been detected. They are classified as members of the family of pseudo-kinases[3]. TRB3 is a mammalian homolog[4] of *Drosphila* tribbles and is also called a neuronal cell death-inducible putative protein kinase gene in rodents. TRB3 is located

on the 20p13 region of the human chromosome[5]. Its full-length translated region in mRNA is 1074 bp, its protein product is made of 358 amino acids and it is a kind of nucleoprotein.

Studies indicate that TRB3 is involved in many biological processes, including insulin resistance (IR), blocking of insulin signaling pathway[6], endoplasmic reticulum stress responses[7] and the regulation of cell growth and differentiation[8,9]. Du *et al*[6] have found that the expression of hepatic TRB3 increased in a rat model of diabetes. It inhibits the activation of the Akt/PKB signaling pathway by insulin, resulting in IR. TRB3 inhibits the phosphorylation of Thr-308 and Ser-473 by binding with them, thus inhibiting the activity of Akt. Then, the insulin signaling pathway is blocked.

Research by Chitturi *et al*[10] indicates that IR exits in about 98% of patients with non-alcoholic fatty liver disease (NAFLD). IR is possibly of key importance in inducing NAFLD. Therefore, any factor related to IR may play an important role in the development of NAFLD. Therefore, TRB3 may not only be a cause of IR, but also an important factor in the occurrence and development of NAFLD. Rat models of NAFLD have been developed by feeding them a high-fat diet. The objective was to study the expression of TRB3 mRNA using reverse transcriptase-polymerase chain reaction (RT-PCR) in rat models of NAFLD, and to evaluate the role of TRB3, using Western blotting analysis, in the occurrence and development of NAFLD.

MATERIALS AND METHODS

Animals

Thirty healthy Sprague-Dawley rats weighing 210-260 g (15 male and 15 female) were purchased from Shanghai Slac Laboratory Animal Co. Ltd., Chinese Academy of Sciences. The rats were fed normal food for 1 wk.

Reagents

The materials for the high-fat-diet rat models and the reagents for pathological tests were all purchased from Shanghai Lanji Technology Development Co., Ltd.; Trizol and SYBR Green I were purchased from Invitrogen. Akt (A444) antibody, p-Akt (S473) antibody and p-Akt (T308) antibody were obtained from Bioworld. Actin and horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG (H + L) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). PVDF membranes were supplied by Millipore. Diaminobenzidine was purchased from Sigma. DEPC-treated Water, TBE and loading buffer were obtained from Shanghai Gene and Biotech Co., Ltd. Taq enzyme and random primers were supplied by Takara Biotechnology (Dalian) Co., Ltd. RNase inhibitor, dNTP, Moloney murine leukemia virus and TEMED were procured from Promega. DTT, SDS, Tris, Glycin, N, N'-Methylene bisacrylamide and acrylamide were obtained from Amresco. BSA was acquired from the Huamei Biotech Company (packaged separately after being imported). Protein markers were purchased by the Shanghai Institute of Biochemistry, Chinese Academy of Sciences. Methyl alcohol was purchased from the Sinopharm Reagent Group (Shanghai, China).

Apparatus

Applied Biosystems 7500 Real-Time PCR System, Vertical Electrophoresis Tank, Electrophoresis Apparatus PAC3000 and Semi-Dry Transfer Unit were purchased from Bio-Rad; high-speed freezing centrifuge, Centrifuge 5417R, was purchased from Eppendorf; Gel Imaging System (GIS)-2008 was purchased from Shanghai Tanon Science & Technology Co., Ltd.; electronic balance BP310P was from Sartorius; pipettes were from Gilson; glass homogenizer was from Ningbo Scientz Scientific Instruments Research Institute; UV-VIS Spectro Photometer Unico UV-2000 was from Unico (Shanghai) Instruments Co., Ltd.; ultrapure water system from Millipore; and ultrasonic cell disruption system Soniprep150 from SANYO.

Animals

The rats were divided into three groups according to random number tables. The control group (n = 10) was fed on a normal diet; NAFLD groups (n = 20) were fed on high-fat diet, which was prepared by adding 10% lard and 2% cholesterol to the normal diet. Group A (n = 10) was fed on high-fat diet for 8 wk and group B (n = 10) was fed on high-fat diet for 16 wk. All the rats lived in an air-conditioned room at room temperature at 18-23℃ and 60% humidity. All the rats were fed *ad libitum* and had free access to water.

Histopathology

According to the schedule of the experiment, the rats were anesthetized with 2.5% pentobarbital sodium solution (1.5 mL/kg), injected into the abdominal cavity after an overnight fasting. They were sacrificed after blood samples were taken from the inferior vena cava, and the livers were removed immediately. Serum and liver paraffin embedded tissue sections were prepared according to routine methods. The liver specimens were fixed in a neutral formalin solution. The tissue sections were hematoxylin and eosin (HE), and the HE-stained sections were examined under a light microscope for the evaluation of hepatic steatosis.

Measurements for observation indexes

Fluorescent quantitative RT-PCR for measurement of TRB3 mRNA expression levels: RNA extraction and cDNA synthesis: The Trizol method was used to extract total RNA from tissues and an UV-VIS Spectrophotometer was used to determine the purity and concentration. Two micrograms of total RNA was reverse transcribed into cDNA.

Real-time fluorescent quantitative PCR: The SYBR

Table 1 Expression levels of TRB3 mRNA, Akt, p-Akt-Thr308, p-Akt-Ser473

Group	Case	TRB3 mRNA (10^{-5})	Akt	p-Akt-Thr308	p-Akt-Ser473
Control	10	3.06 ± 2.33	1.03 ± 0.53	0.92 ± 0.38	0.45 ± 0.26
A	10	5.77 ± 4.20	1.12 ± 0.77	0.82 ± 0.45	1.48 ± 0.50[b]
B	10	122.28 ± 95.37[b]	0.41 ± 0.16[b]	0.47 ± 0.19[b]	0.81 ± 0.39[c]

[b]$P < 0.01$ *vs* control group/group A; [c]$P < 0.05$ *vs* group A.

Green I dye method was adopted. GADPH and TRB3 were amplified by reverse transcription. After gel electrophoresis of amplified products, a fully-automatic Gel Imaging System was used to analyze mRNA expression to compare the intensity between the groups. All the results were normalized to GADPH. The GADPH primers were used as follows: upstream primer: 5'-ACCACAGTCCATGCCATCAC-3', downstream primer: 5'-TCCACCACCCTGTTGCTGTA-3'. The length of the amplified product was 440 bp. The TRB3 primers were used as follows: upstream primer: 5'-TCA TCTTGCGCGACCTCAA-3', downstream primer: 5'-TCCACCACCCTGTTGCTGTA-3'. The length of the amplified product was 296 bp. Thirty-six cycles of pre-degeneration at 95℃ for 2 min, degeneration at 95℃ for 10 s, annealing at 50℃ for 10 s, and extension at 72℃ for 45 s were used for all experiments.

Western blotting analysis for the expression levels of total Akt and phosphorylated Akt (p-Akt-Thr308, p-Akt-Ser473): Equal samples of tissue were prepared and put into protein extracts to be ground as plasm form. Then the plasm was high-speed centrifuged under freezing conditions for protein extraction. The protein concentration was determined according to the fixed steps. The protein samples (30 μL) were subjected to SDS-PAGE electrophoresis, transferred to PVDF membranes, and shaken on a rotary shaker at room temperature for 2 h. After that, a TBST buffer solution was used to wash the membrane three times. Then, Akt-related antibodies (Akt1, p-Akt-Thr308, p-Akt-Ser473) were incubated at 4℃ overnight under constant shaking on a rotary shaker. After three washes with TBST buffer solution, HRP-labeled goat anti-rabbit IgG (H + L) was incubated at room temperature while shaking on a rotary shaker for 2 h. NBT/BCIP reagent was applied for color development. The membrane was rinsed with deionized water. All the procedures were repeated three times. β-actin was chosen as an internal control. The GIS was used for data analysis, and statistical analysis was used to detect differences between samples and the internal control.

Statistical analysis

SPSS11 software was used for the statistical analysis. All the statistical data were expressed as mean ± SD for single factor analysis of variance and paired comparisons were performed by the least-square deconvolution method. Two-tailed tests ($\alpha = 0.05$) were used for statistical treatment. $P < 0.05$ was considered a significant difference.

RESULTS

Histopathological changes

A high-fat diet causes obvious hepatic steatosis in rats, which was evident for both model groups. Mild to moderate hepatic steatosis occurred in group A, mainly as mild steatosis. Moderate to severe hepatic steatosis occurred in model group B, mainly as severe steatosis. Mild hepatic steatosis tissue was defined as hepatic steatosis that accounted for 30%-50% of the total liver cells in the microscopic field; for moderate hepatic steatosis, liver cells with hepatic steatosis accounted for 50%-75% of the total liver cells; and for severe hepatic steatosis tissues, liver cells with hepatic steatosis accounted for over 75% of the total liver cells. In portal areas, severe inflammation featured infiltration of large numbers of diffuse lymphocytes and neutrophils, destroyed limiting plates and hepatic lobules were infiltrated by inflammatory cells that surrounded liver cells (Figure 1).

Fluorescent quantitative RT-PCR for measurement of TRB3 mRNA expression levels

We adopted real-time fluorescent quantitative RT-PCR methods to measure the expression levels of TRB3 mRNA in rats (Figure 2A-C). The relation ($r = 0.99$) between amplified results of PCR and Ct value of standard samples is shown in Figure 2C. From the melting curve, no primer dimer formation was detected during the PCR reaction (Figure 2B). We found that the expression level of TRB3 mRNA in group B was significantly higher than in the control group (122.28 ± 95.37 *vs* 3.06 ± 2.33, $P = 0.001$) and group A (122.28 ± 95.37 *vs* 5.77 ± 4.20, $P = 0.001$). There was no significant difference (5.77 ± 4.20 *vs* 3.06 ± 2.33, $P = 0.914$) in the expression levels of TRB3 between group A and the control group (Figure 3, Table 1). All these data indicate that a simple hepatic steatosis pathomorphism showed no significant difference between group A and the control group for the expression of TRB3 mRNA. As the time to set up the model increased, the degree of hepatic steatosis was raised. When the model deteriorated to show evidence of a fatty hepatitis pathomorphism, the expression of TRB3 mRNA was significantly higher.

Western blotting analysis for the expression levels of total Akt and phosphorylation Akt (p-Akt-Thr308, p-Akt-Ser473)

The protein bands of Akt p-Akt-Thr308 and p-Akt-

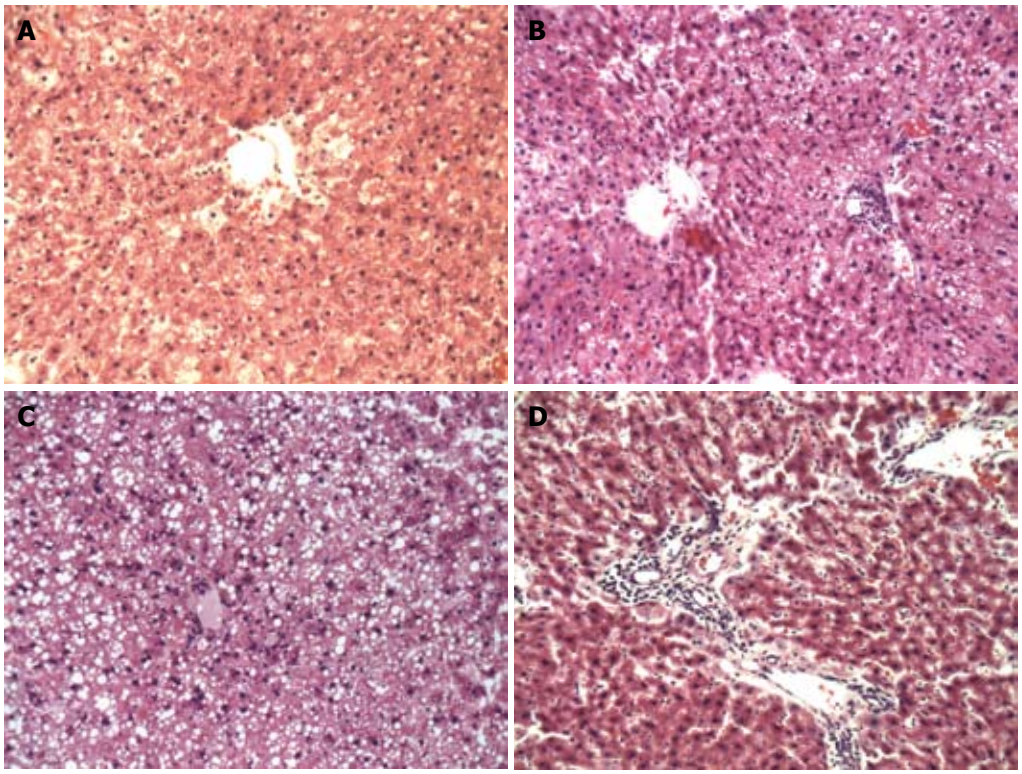

Figure 1 The degree of hepatic steatosis in each group. A: In group A, mild hepatic steatosis was observed on histological examination (HE, × 200); B: In group A, moderate hepatic steatosis was observed (HE, × 200); C: In group B, hepatocytes showed severe hepatic steatosis. Liver cells with hepatic steatosis accounted for over 75% of the total liver cells (HE, × 200); D: In group B, hepatocytes showed severe steatosis along with portal inflammation (HE, × 200).

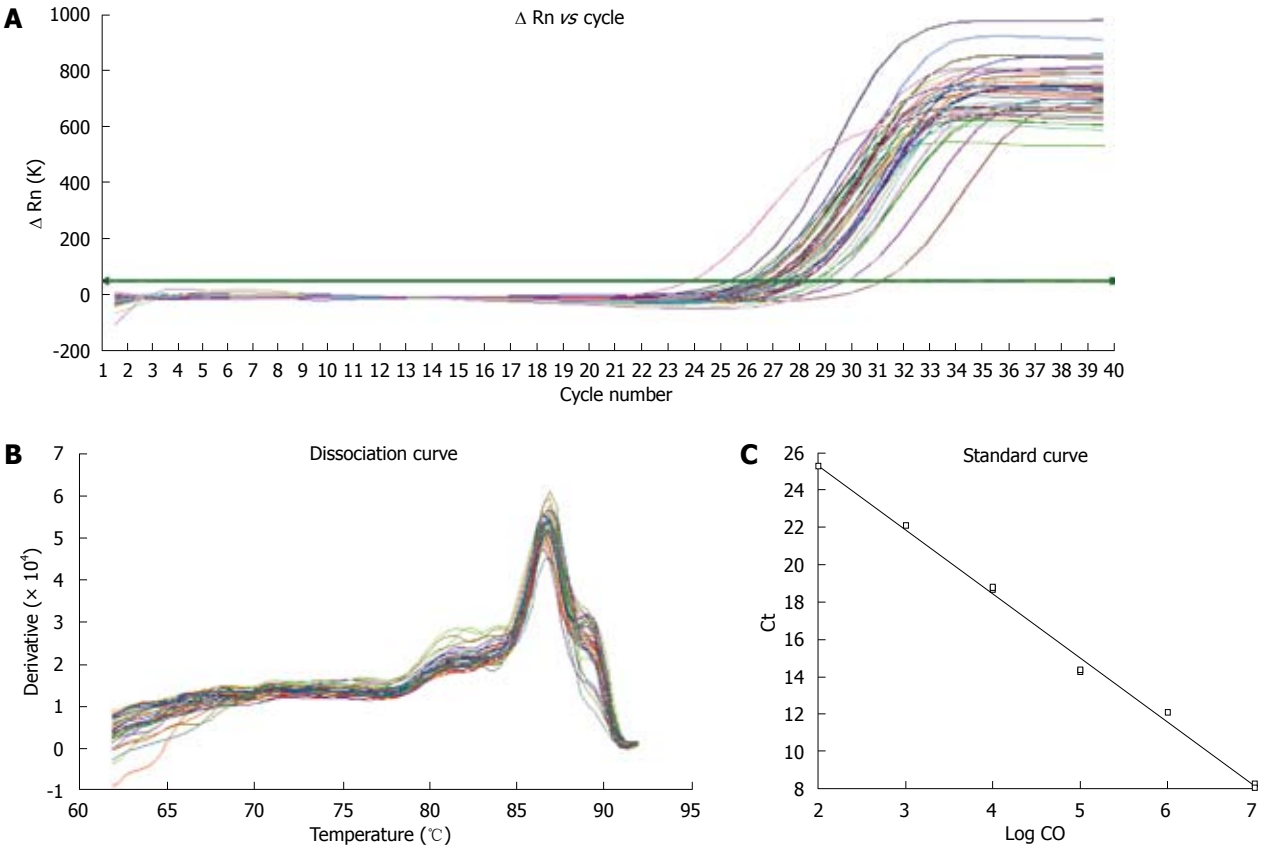

Figure 2 Fluorescent quantitative PCR for measurement of TRB3 mRNA expression levels. Real-time PCR was used for absolute quantification analysis. A: Amplification curve with the threshold value set in the exponential growth phase; B: Melting curve showing no primer dimer in PCR reaction; C: Standard curve for the relation (r = 0.99) between amplified results of PCR and Ct value of standard samples was in accordance with the requirements of real-time PCR. The intensities of fluorescent signals indicated the variation of product concentrations.

Ser473 are shown for each group. There was no significant difference in the expression levels of Akt (1.03 ± 0.53 *vs* 1.12 ± 0.77, P = 0.729) and p-Akt-Thr308 (0.82 ± 0.45 *vs* 0.92 ± 0.38, P = 0.592) between group A and

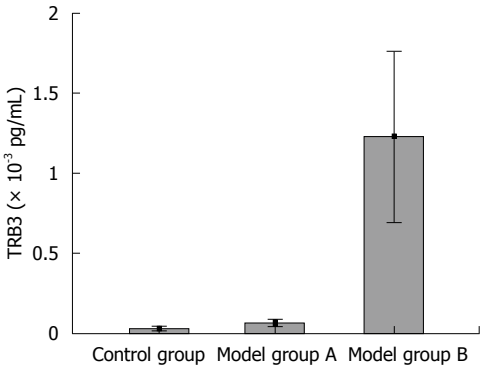

Figure 3 Comparison of TRB3 expression levels among the three groups. The TRB3 mRNA expression level in group B was significantly ($P < 0.01$) higher than in the control group and group A. There was no significant difference ($P > 0.05$) between group A and the control group.

the control group. However, the expression levels of Akt and p-Akt-Thr308 in group B was significantly lower than in group A (Akt 0.41 ± 0.16 *vs* 1.12 ± 0.77, $P = 0.008$; p-Akt-Thr308 0.47 ± 0.19 *vs* 0.82 ± 0.45, $P = 0.036$) and the control group (Akt 0.41 ± 0.16 *vs* 1.03 ± 0.53, $P = 0.018$; p-Akt-Thr308 0.47 ± 0.19 *vs* 0.92 ± 0.38, $P = 0.010$). The expression level of p-Akt-Ser473 in group A was significantly higher than in group B (1.48 ± 0.50 *vs* 0.81 ± 0.39, $P = 0.041$) as well as the control group (1.48 ± 0.50 *vs* 0.45 ± 0.26, $P = 0.003$) (Table 1). All these data indicate that a simple hepatic steatosis pathomorphism does not produce a significant difference between the model group and the control group in the expression of total Akt. As the rats were exposed to longer periods of a high-fat diet, the degree of hepatic steatosis was increased. When the rats deteriorated to a fatty hepatitis pathomorphism, the expression of total Akt, p-Akt-Thr308 and p-Akt-Ser473 was significantly lower than the simple fatty liver disease modeled by group A and the control group.

DISCUSSION

NAFLD has increased in recently years, and is one of the major causes for cryptogenic cirrhosis[11,12]. The pathogenesis of NAFLD is complicated. There is an interaction between genetic susceptibility and multiple metabolic disorders involved in the disease. The pathophysiologic basis of the condition is mainly insulin resistance and oxidative stress. No perfect theory exists for all its clinical manifestations[13,14]. At present, IR along with hepatocyte fatty degeneration is believed to be a key factor in the occurrence and development of fatty liver disease[15,16]. Research indicates[10] that IR exits in about 98% of patients with NAFLD. IR is probably of key importance for the induction of NAFLD. Therefore, any factor related to IR may play an important role in NAFLD.

Studies indicate that TRB3 is involved in many biological processes, including IR and blocking of the insulin signaling pathway[6,17]. In this study, we generated a rat model of NAFLD using a high-fat diet. Group A modeled simple fatty liver, and we continued to feed the rats with the high-fat diet. Simple hepatic steatosis produced no significant difference between the model group and the control group in the expression of TRB3 mRNA. As the rats were exposed to longer periods with the high-fat diet, the extent of hepatic steatosis was raised. When the condition of the rats deteriorated to a fatty hepatitis pathomorphism (group B), the expression of TRB3 mRNA became significantly higher. This result indicates that TRB3 is involved in the occurrence and development of NAFLD.

On the basis of recent research, through this study, we made further efforts to discover the possible mechanism of TRB3 involved in the occurrence and development of NAFLD. One study has shown that IR is related to the insulin signaling pathway phosphoinositide 3-kinase/protein kinase B (PI3-K/PKB)[18]. Akt is a key protein[19] in the PI3-K insulin signaling pathway. Two of its sites need to be phosphorylated[20,21] for its normal physiological function. One site is Thr-308, located in the kinase domain, and the other one is Ser-473, located in hydrophobic motif of the regulatory domain. After the binding of insulin and its receptors in the cell membrane, the upstream signaling proteins in this pathway are activated step by step. Thr-308 and Ser-473[22,23] phosphorylation sites for Akt are activated, and then endocytose from membrane to cytoplasm, which starts a cascade of reactions of the downstream related substrate proteins. Through this process, insulin contributes to glycogen synthesis, glucose transport, glycolysis and the inhibition of gluconeogenesis[24]. The quantity of Akt decreases and its activity changes in rats with IR[25]. Ijuin *et al*[26] have found that TRB3 may inhibit the signal transduction of insulin-activated PI3-K in CHO cells, which suggests that TRB3 affects insulin signal transduction and inhibits uptake and utilization of glucose by cells. Du *et al*[6] and Matsushima *et al*[27] have found that in the hepatic cells of TRB3 transgenic rats, TRB3 inhibited the phosphorylation and activation of Thr-308 and Ser-473 of Akt, but did not affect the protein expression of Akt. Therefore, TRB3 may decrease glucose tolerance and cause blood glucose elevation. The phosphorylation of substrate proteins like glycogen synthase kinase-3β by Akt was inhibited, and glycogen synthesis and the function of insulin on glucose metabolism was also lowered. TRB3 plays an important role in IR. TRB3 gene knockouts may increase the sensitivity of hepatic cells to insulin stimulation. The activity of Akt may be enhanced, and blood glucose levels may be lower[28]. The above mentioned studies indicate that TRB3 could block the insulin signaling pathway through inhibiting Akt activation[6]. Since TRB3 inhibits the phosphorylation of Thr-308 and Ser-473 by binding with them, it inhibits the activity of Akt. As a result, the insulin signaling pathway is blocked. Our results also support this hypothesis. When the pathomorphism was simple hepatic steatosis, there was no significant difference between the model group and control group in the expression of TRB3

mRNA. The expression level of total Akt did not change much either. As the degree of hepatic steatosis was raised and deteriorated to fatty hepatitis, the expression of total Akt, p-Akt-Thr308 and p-Akt-Ser473 was significantly lower than that in the simple fatty liver model group.

The data for p-Akt-Ser473 in Table 1 show that in mild steatosis (group A), expression levels are much greater than control while in severe steatosis (group B), levels go back to the control value. We assume that the complex regulation of active molecules *in vivo* may lead to another pathway. Balendran *et al*[29] have shown that 3-phosphoinositide-dependent protein kinase-1, Akt and protein-kinase-C-related kinase-2 interact with each other after the phosphorylation of Thr308, which can be converted into 3-phosphoinositide-dependent protein kinase-2 (PDK2) and modify Ser473. Kroner *et al*[30] have found that the existence of PDK2 can be proven by the complex relationship between Thr308 and Ser473, which is phosphorylated independently. A study by Ferguson *et al*[31] has shown that Akt can be activated in a PI3-K-independent pathway. Therefore, this interesting phenomenon is worthy of further study.

In conclusion, TRB3 can block the insulin signaling pathway by inhibiting the activation of Akt[32,33], and contributing to IR. Therefore, TRB3 may be an important factor in the occurrence and development of NAFLD. This study provides an experimental basis for future studies about the role of TRB3 in NAFLD. The control of the expression level of TRB3 in liver may become a new target for NAFLD therapy.

COMMENTS

Background

Tribble 3 (TRB3) is involved in many biological processes, including insulin resistance (IR), blocking of the insulin signaling pathway, endoplasmic reticulum stress responses, and the regulation of cell growth and differentiation. Any factor related to IR will play an important role in the development of non-alcoholic fatty liver disease (NAFLD). Therefore, the authors of this study investigated the relationship between TRB3 and IR, and aimed to establish its importance in the occurrence and development of NAFLD.

Research frontiers

The study is the first to evaluate the role of TRB3 with IR in NAFLD. The potential effect of TRB3 is likely to block the insulin signaling pathway through inhibiting Akt activation. TRB3 may play an important role in the occurrence and development of NAFLD.

Innovations and breakthroughs

This study explained one of the possible mechanisms of IR, which could produce a potentially facilitative effect on the occurrence and development of NAFLD.

Applications

This study provides an experimental basis for future studies on the role of TRB3 in NAFLD. The control of the expression level of TRB3 in liver may become a new target for therapy for NAFLD.

Peer review

In the present study, the authors tested the effect of TRB3 in NAFLD in rats, and found a facilitative effect in the occurrence and development of NAFLD.

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BRIEF ARTICLES

Non-steroidal anti-inflammatory drugs and statins in relation to colorectal cancer risk

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Abstract

AIM: To investigate the association between individual or combined use of non-steroidal anti-inflammatory drugs (NSAIDs) or statins and colorectal cancer risk.

METHODS: In a population-based case-control study in women, we examined the association between NSAIDs and statin use and the risk of colorectal cancers. We further investigated whether the use of statins modifies the protective effect of NSAIDs. Female cases (n = 669) of colorectal cancer aged 50-74 years were identified from a statewide registry in Wisconsin during 1999-2001. Community control women (n = 1375) were randomly selected from lists of licensed drivers and Medicare beneficiaries. Medication use and risk factor information were gathered during a structured telephone interview. A multivariable logistic regression model was used to calculate odds ratio (OR) and 95% confidence interval (CI).

RESULTS: Overall, NSAIDs users had a 30% reduction in risk of colorectal cancer (95% CI: 0.56-0.88). Statin use was not associated with colorectal cancer risk (OR = 1.17, 95% CI: 0.74-1.85), regardless of structural type (lipophilic or hydrophilic), duration of use, or recency. There was no evidence of an interaction between NSAIDs and statins and colorectal cancer risk (P-interaction = 0.28).

CONCLUSION: Although our results confirm the inverse association between NSAIDs use and colorectal cancer risk, they do not support a risk reduction in statin users, or an interaction effect of combined NSAIDs and statin use.

Key words: Non-steroidal anti-inflammatory drugs; Statin; Colorectal cancer; Cancer prevention; Chemoprevention

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INTRODUCTION

There is strong evidence for a reduced risk of colorectal cancer in regular users of non-steroidal anti-inflammatory drugs (NSAIDs)[1,2] and some promising but inconsistent observational data regarding a role of statins in this risk[3-11]. An interaction between the use of NSAIDs and statin on the risk of colorectal cancer is suggested by both *in vivo* and *in vitro* studies[5,12,13].

NSAIDs induce apoptosis in colon cancer cells[14,15]. By blocking cyclooxygenase enzymes, they also inhibit prostaglandin production, which is known to promote tumor angiogenesis and cell proliferation[1]. Statins have anti-neoplastic effects through both HMG-CoA (3-hydroxy-3-methyl-glutaryl-CoA) dependent and independent processes[13,16,17]. Inhibition of the prenylation of cell signaling proteins, as well as the anti-inflammatory and anti-oxidative properties of statins, are thought to be responsible for their anti-cancer effects[16]. Augmentation of sulindac or celecoxib induced apoptosis by Lovastatin in colon cancer cell lines and

the increased activation of caspase-3, a pro-apoptotic protein, in combined statin and NSAIDs use[12,18], suggest a synergistic anti-cancer effect. These observations have also been supported by some observational data[5].

The purpose of this study was to investigate the effects of NSAIDs and statin use in relation to colorectal cancer in a population-based case-control study in women. We also investigated whether the use of statins modified the relationship between NSAIDs and statins.

MATERIALS AND METHODS

Female cases (n = 669) of colorectal cancer aged 50-74 years were identified from the Wisconsin cancer reporting system, the statewide tumor registry, during 1999-2001. Registry reports included stage, histology and limited treatment information. Of the 1038 eligible cases, 170 (16.4%) were deceased, 19 (1.8%) were not contacted due physicians' disapproval, 22 (2.1%) could not be located and 154 (14.8%) declined to participate, resulting in a 65% response rate. We also excluded four cases with unreliable interviews. Community control (n = 1375) women were randomly selected to match the age distribution of cases from two sampling frames: lists of licensed drivers (age < 65 years) and Medicare beneficiaries (age ⩾ 65 years). Women were ineligible as controls if they reported a history of colorectal cancer. The response rate for controls was 79%.

Structured telephone interviews were conducted to obtain information regarding medication use, including NSAIDs and statins, and other factors (Table 1). We considered the most commonly used statins that were approved by the Food and Drug Administration from 1995 through 2000. Having ever used NSAIDs or statins was confined to subjects who reported using the medications for at least 30 d. We defined the duration of each period of NSAIDs or statin use. Use of these preparations within one year before the reference year was considered as current use. We categorized statins according to whether they were lipophilic (simvastatin, lovastatin and fluvastatin) or hydrophilic (pravastatin), as it has been suggested that the anti-cancer activity of statins might be limited to the ones with lipophilic structure[16].

Odds ratios (OR) and 95% confidence intervals (CI) were calculated from multivariable logistic regression models to estimate the associations between NSAIDs and statins with the risk of colorectal cancer. We also evaluated possible interaction between NSAIDs and statin use by including a cross-product term of "ever use" of these medications in the regression model. We adjusted for the potential confounding factors (Table 1) by including them in the multivariate models.

RESULTS

Overall, 657 cases of colorectal cancer and 1342 controls were included in the analysis (Table 1). The prevalence of regular NSAIDs use in the sample was 33% (20% aspirin and 13% non-aspirin, 26% current users). The prevalence of statin use was 7% (6% current users, 5% lipophylic and 2% hydrophylic) (Table 2).

Table 1 Characteristics of women with colorectal cancer and controls *n* (%)

Characteristic	Cases (n = 657)	Controls (n = 1342)[1]
Education		
No high school diploma	95 (14.8)	119 (11.8)
High school diploma	312 (48.7)	632 (50.0)
Some college	143 (22.3)	312 (20.7)
College degree	91 (14.2)	257 (17.6)
Type of postmenopausal hormone therapy		
Never	417 (65.2)	696 (55.7)
Estrogen only	73 (11.4)	145 (10.9)
Estrogen and progestin only	41 (6.4)	133 (7.2)
Other combination	109 (17.0)	344 (26.2)
Family history of colorectal cancer		
No	492 (80.9)	1060 (87.2)
Yes	116 (19.1)	171 (12.8)
Body mass index (kg/m^2)		
< 25	273 (42.7)	538 (40.9)
25-30	206 (32.2)	465 (36.9)
⩾ 30	160 (25.0)	314 (22.1)
History of colorectal cancer endoscopic screening (colonoscopy/ sigmoidoscopy)		
No	429 (67.3)	816 (61.5)
Yes	208 (32.6)	455 (38.5)
Smoking history (pack-years)		
Never	311 (48.7)	677 (54.7)
< 10	101 (15.8)	208 (15.1)
10-20	53 (8.3)	127 (7.4)
⩾ 20	174 (27.2)	305 (22.9)

[1]Control percentages were age-adjusted to the cases age distribution. In this table, percentages are based on excluding unknowns in that category.

Those who had ever used NSAIDs had a 30% decrease in colorectal cancer risk (OR = 0.70; 95% CI: 0.56-0.88) compared to those who had never used NSAIDs. The risk reduction was statistically significant in current users but not in former users and there was no trend for increasing duration (P = 0.75).

Having ever used statins was not associated with colorectal cancer risk (OR = 1.17; 95% CI: 0.74-1.85) regardless of the type of statin (lipophilic or hydrophilic). Neither long term (> 3 years) nor current statin use were associated with risk.

Having ever used both NSAIDs and statins was not associated with colorectal cancer risk (OR = 0.96; 95% CI: 0.49-1.78). The association between NSAIDs use and colorectal cancer risk was not modified by use of statins (P-interaction = 0.28) (data not shown).

DISCUSSION

Our finding of a 30% reduced risk of colorectal cancer with NSAIDs use is consistent with the current evidence. The observed colorectal cancer risk reductions range from 20% to 40%, possibly due to the heterogeneity of study designs[1].

In contrast to our findings on NSAIDs use, we did not observe an association between statin use and colorectal cancer risk. This association has been examined in secondary analyses of randomized controlled trials that

Table 2 Multivariable OR of colorectal cancer associated with statin and NSAIDs use

	Cases *n* (%)	Controls *n* (%)	OR[1]	95% CI[1]	OR[2]	95% CI[2]
NSAIDS						
Never	462 (71.9)	837 (63.6)	1.00	Reference	1.00	Reference
Ever	181 (28.1)	480 (36.4)	0.69	0.55-0.86	0.70	0.56-0.88
Former	41 (6.4)	109 (8.3)	0.74	0.50-1.11	0.77	0.51-1.15
Current	140 (21.8)	371 (28.2)	0.68	0.53-0.86	0.68	0.53-0.88
Duration (yr)						
< 1	8 (1.2)	25 (1.9)	0.71	0.30-1.65	0.71	0.30-1.69
1-4	85 (13.2)	233 (17.7)	0.70	0.52-0.93	0.70	0.52-0.94
⩾ 5	88 (13.7)	222 (16.9)	0.68	0.51-0.92	0.71	0.52-0.96
Statins						
Never use	453 (92.6)	1114 (93.2)	1.00	Reference	1.00	Reference
Ever use	36 (7.4)	81 (6.8)	1.03	0.66-1.60	1.17	0.74-1.85
Former	4 (0.8)	9 (0.8)	1.63	0.49-5.44	1.93	0.56-6.06
Current	32 (6.5)	72 (6.0)	0.97	0.60-1.55	1.09	0.67-1.78
Duration (yr)						
< 3	17 (3.5)	41 (3.4)	0.96	0.51-1.80	1.07	0.56-2.03
⩾ 3	19 (3.9)	40 (3.3)	1.10	0.60-2.00	1.27	0.68-2.38
Type						
Lipophilic use	30 (6.1)	63 (5.3)	1.04	0.64-1.70	1.20	0.72-2.00
Hydrophilic use	7 (1.4)	20 (1.7)	1.06	0.43-2.63	1.10	0.44-2.77

[1]Adjusted for age and reference year. [2]Adjusted for age, reference year, education, post menopausal hormone use, first degree family history of colorectal cancers, body mass index, history of colorectal cancer endoscopic screening, and smoking.

did not show a risk reduction among users[19]. The small number of colorectal cancer cases should be considered while interpreting these trial results as they were designed to measure cardiovascular outcomes. Observational studies have also produced inconsistent findings. While two case-control studies[5,10] reported risk reduction in long term statin users, other studies[3,4,6,9,10] did not show such an inverse association[20-25]. In a large case-controlled study[10] of 1953 cases and 2015 controls, a 50% reduction in colorectal cancer risk (OR = 0.53; 95% CI: 0.38-0.74) was observed in long term statin users (more than 5 years).The difference between databases from which the cases and controls were selected might have influenced the results. In their study, all the incident cases from northern Israel were included, while controls were recruited from a health maintenance organization, possibly making them more likely to have a healthier life style. In another population-based case-controlled study conducted in Germany[5] (537 cases and 612 controls), a 35% risk reduction (OR = 0.65; 95% CI: 0.43-0.99) was observed among statin users. However, after adjustment for NSAIDs use, the estimate did not remain statistically significant.

We also did not find any combined effect for NSAIDs and statins. To our knowledge, only two other population-based studies[3,5] have looked at the combined effect of NSAIDs and statins on colorectal cancer risk. While one[5] suggested a stronger risk reduction in combined users than we hypothesized, neither found evidence of a statistically significant interaction between NSAIDs and statin use (*P* interactions = 0.37 and 0.21, respectively).

Statin use was uncommon in our study subjects, which may have limited our ability to detect a true reduced risk. However, in another study from our group[26], with a similar design and population, a significant reduction in breast cancer risk was observed only among regular users of fluvastatin, which also had low prevalence of use. Statins are relatively new medications, therefore examining outcomes like adenomatous polyps as an intermediate step in colorectal cancer development might be a reasonable approach to evaluate both individual and combined effect of statins on colorectal cancer risk. Our study was restricted to women, but there are no reported gender effects on the association of drugs with colorectal cancer risk. The availability of detailed information, control for potential confounding factors, and reliable exposure measurements are the major strengths of our study.

In conclusion, these results support the inverse association between NSAIDs use and colorectal cancer risk in women, especially in current users. We did not detect an association between colorectal cancer risk and statin use, regardless of type (lipophilic *vs* hydrophilic), recency or duration of use. Further, there was no interaction effect of combined NSAIDs and statin use.

COMMENTS

Background

The use of non-steroidal anti-inflammatory drugs (NSAIDs) such as Aspirin is known to be inversely associated with risk of developing colorectal cancer. Some studies have suggested such an association with the use of the commonly used lipid lowering drugs, statins. There is also some experimental data suggesting a synergistic effect for these two popular drug families against colorectal cancer risk.

Research frontiers

While NSAIDs have some possible protective effect against colorectal cancer, they are not yet approved for routine use for this purpose, mainly because of their potentially fatal side effect, bleeding. Finding another protective agent that works synergistically with NSAIDs, allowing a decreased NSAIDs dose, could lower the incidence of the side effect whilst preserving the desired effect; cancer prevention. The promising evidence indicating such an effect for statins is exciting, because these drugs are a hot topic for different preventive strategies, especially in cardiovascular diseases.

Applications

The study results confirm the previously known inverse association between NSAIDs use and colorectal cancer risk.

Peer review

This is a retrospective case-controlled study investigating if NSAIDs or/and statins have chemopreventive effects in women with regard to colorectal cancer (CRC). It is well known that regular users of NSAIDs are at less risk of developing gastrointestinal cancers, including CRC. This paper supports this hypothesis.

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BRIEF ARTICLES

Study of the patency of different peritoneal drains used prophylactically in bariatric surgery

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Abstract

AIM: To compare the performance of different types of abdominal drains used in bariatric surgery.

METHODS: A vertical banded Roux-en-Y gastric bypass was performed in 33 morbidly obese patients. Drainage of the peritoneal cavity was performed in each case using three different types of drain selected in a randomized manner: a latex tubular drain, a Watterman tubulolaminar drain, and a silicone channeled drain. Drain permeability, contamination of the drained fluid, ease of handling, and patient discomfort were evaluated postoperatively over a period of 7 d.

RESULTS: The patients with the silicone channeled drain had larger volumes of drainage compared to patients with tubular and tubulolaminar drains between the third and seventh postoperative days. In addition, a lower incidence of discomfort and of contamination with bacteria of a more pathogenic profile was observed in the patients with the silicone channeled drain.

CONCLUSION: The silicone channeled drain was more comfortable and had less chance of occlusion, which is important in the detection of delayed dehiscence.

Key words: Bariatric surgery; Contamination; Drains; Gastric leak; Patency; Peritonitis

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INTRODUCTION

Most of the immediate complications occurring after bariatric surgery are due to technical errors that may go unrecognized[1]. Among them, intraoperative bleeding and dehiscence of anastomoses, although infrequent, are the most feared complications. Dehiscence occurs at a frequency of 1% to 4.4% of cases, resulting in significant morbidity and eventually even death. The early detection of this complication could reduce morbidity and mortality[2-4].

Resources for the early diagnosis of dehiscence during the postoperative period are limited. The clinical signs and symptoms are difficult to interpret and imaging exams, when they can be performed, may yield false results due to excess body weight.

Over the last three decades, efforts have been made to investigate the effectiveness of prophylactic drainage of the peritoneal cavity in controlled randomized clinical trials[5-8]. Although there are no evidence-based data justifying the use of drains in various situations, including bariatric surgery, most services routinely use them for the early identification of fistulae and their treatment[9].

Different types of drains are available but the search is ongoing for the ideal model. A closed-system model of a silicone drain was recently produced, with multiple channels in its intra-abdominal portion and vacuum

aspiration (Blake®-Ethicon), which has been used in various operations including bariatric surgery[10-16].

The objective of the present study was to assess the patency of three different types of abdominal drains used in bariatric surgery.

MATERIALS AND METHODS

During the period from January to September 2007, 33 morbidly obese patients were selected for surgical treatment by banded Roux-en-Y gastric bypass. The patients were divided into three groups according to the type of drain employed in the peritoneal cavity. The study was approved by the Research Ethics Committee of the hospital and all patients gave written informed consent to participate.

The type of drain used was selected at random: Group 1, closed-system latex tubular drain with multiple holes and without aspiration; Group 2, Watterman drain consisting of two No. 16 Levin catheters with multiple holes wrapped with a No. 4 Penrose tube (open system) (Figure 1); Group 3, silicone drain with multiple channels (Blake®Ethicon) 24 Fr connected to a 300 mL J-Vac® reservoir (Ethicon) under continuous vacuum. All drains were left in place for seven days after surgery.

Before removal of the drain, the patient received 120 mL of a methylene blue solution by the oral route in order to test for the presence of possible anastomosis dehiscence of staple lines. No radiological test was applied. For the evaluation of drain permeability, the daily output of each drain was recorded over a postoperative period of seven days.

Microbiological and antimicrobial analysis of the intraperitoneal end of the drains was performed on the seventh postoperative day during the interruption of drainage. In order to obtain peritoneal fluid the drains were punctured in their external portion. The end of each drain located in the peritoneal cavity was also sent for analysis. Both procedures were carried out under rigorous asepsis.

Subjective evaluation of the comfort of each drain was performed using a questionnaire which was completed by the patient on the day of drain removal. The information obtained referred to pain at the drain site and to pain during drain removal (graded from 0 to 5), ease of handling and discomfort with the presence of odors.

Groups were compared by one way analysis of variance (ANOVA) and then paired for application of the Tukey post-test. The level of significance was set at 5%.

RESULTS

All patients who underwent surgery were evaluated. Mean patient age, weight and BMI were 37.1 years, 138.20 kg and 51.42 kg/m^2, respectively. The characteristics of the groups studied were similar (Table 1).

All patients had a favorable postoperative course without major complications. There was no extravasation of methylene blue during the tests carried out on the seventh postoperative day. However, the intraperitoneal end of the Watterman drain in a group 2 patient was stained blue at the time of removal. A No. 16 Levin catheter was immediately introduced in this patient in order to maintain patency. No significant drainage occurred on subsequent days and the patient's course was favorable.

Table 1 Individual characteristics of the experimental groups

	Group 1-Latex	Group 2-Watterman	Group 3-Blake
Age (yr)	35.45 ± 7.56	36.18 ± 10.68	39.81 ± 9.52
Gender: M/F	3/8	3/8	1/10
Weight (kg)	138.48 ± 17.58	135.98 ± 19.86	140.30 ± 24.58

Data are reported as mean ± SD.

Table 2 Volume of liquid collected daily with each type of drain

Postoperative days	Group 1 Latex	Group 2 Watterman	Group 3 Blake	*P*
Day 1	146 ± 57.5	190 ± 178.6	150 ± 79	0.656
Day 2	89 ± 74.1	96.7 ± 68.8	168.2 ± 107.6	0.091
Day 3	29.4 ± 27.7	57.3 ± 53	107.5 ± 79.2	0.016
Day 4	25.3 ± 16.6	34.8 ± 39.3	106.2 ± 106.5	0.021
Day 5	28.3 ± 40.7	34.1 ± 30.4	123.5 ± 105.3	0.005
Day 6	26.8 ± 40.6	21.3 ± 17	88.5 ± 51.8	0.001
Day 7	26.9 ± 36	19.5 ± 18.6	89.7 ± 76	0.007

Results in milliliters and represented by mean ± SD. *P* value obtained by ANOVA.

Table 3 Paired comparison between the drains, regarding the drained volumes

Postoperative days	Blake *vs* Watterman	Blake *vs* Latex	Latex *vs* Watterman
Day 1	$P > 0.05$	$P > 0.05$	$P > 0.05$
Day 2	$P > 0.05$	$P > 0.05$	$P > 0.05$
Day 3	$P > 0.05$	$P < 0.05$	$P > 0.05$
Day 4	$P < 0.05$	$P < 0.05$	$P > 0.05$
Day 5	$P < 0.05$	$P < 0.05$	$P > 0.05$
Day 6	$P < 0.01$	$P < 0.01$	$P > 0.05$
Day 7	$P < 0.05$	$P < 0.05$	$P > 0.05$

P value obtained by the Tukey post-test.

Drain output

No difference in collected fluid volume was observed up to the second postoperative day. Starting on the third day, the silicone channeled drain showed significantly greater drainage compared to the others (Table 2) and this difference persisted up to the 7th postoperative day. No difference in collected volume was observed between the tubular latex drain and the tubulolaminar (Watterman) drain (Table 3).

Microbiological analysis

Microbiological evaluation of the fluid from the peritoneal cavity collected through the various drains revealed that nine patients with the silicone channel drain had a positive culture, with the bacteria most frequently detected being *Staphylococcus* spp., *Proteus* spp. and *Klebsiella* spp.; for the

Table 4 Microbiology of the fluid drained from the peritoneal cavity and from a part of the intraperitoneal segment of the drain

	Group 1-Latex	Group 2-Watterman	Group 3-Blake
Patient 1	*Pseudomonas aeruginosa*	*Enterobacter cloacae*[1]	*Staphylococcus aureus*
	Pseudomonas aeruginosa	*Escherichia coli*[1]	*Staphylococcus aureus*
Patient 2	*Enterobacter aerogenes*	*Proteus mirabilis*	*Proteus mirabilis*
	Enterobacter aerogenes	*Proteus mirabilis*	*Pseudomonas aeruginosa + Morganella morgani*
Patient 3	*Klebsiella pneumoniae + Staphylococcus simulans*	*Serratia marcescens*	*Staphylococcus aureus + Proteus mirabilis + Enterobacter cloacae*
	Staphylococcus aureus + Klebsiella pneumoniae + Morganella morgani + Proteus mirabilis	*Serratia marcescens*	*Serratia marcescens + Enterococcus faecalis*
Patient 4	*Serratia marcescens*	*Pseudomonas aeruginosa*	*Proteus mirabilis + Klebsiella pneumoniae + Enterococcus faecalis*
	Serratia marcescens	*Pseudomonas aeruginosa*	*Staphylococcus aureus + Proteus mirabilis*
Patient 5	*Escherichia coli + Proteus mirabilis*	*Enterobacter cloacae*	*Proteus mirabilis*
	Pseudomonas aeruginosa	*Klebsiella pneumoniae*	*Proteus mirabilis*
Patient 6	*Citrobacter koseri*	*Proteus vulgaris*	*Klebsiella pneumoniae + Proteus mirabilis*
	Proteus mirabilis + Citrobacter koseri	*Proteus vulgaris*	*Klebsiella pneumoniae + Proteus mirabilis*
Patient 7	*Staphylococcus aureus + Proteus mirabilis*	*Pseudomonas aeruginosa + Klebsiella pneumoniae*	-
	Staphylococcus aureus + Proteus mirabilis	*Pseudomonas aeruginosa*	-
Patient 8	*Proteus mirabilis*	*Enterococcus faecalis + Staphylococcus aureus*	-
	Proteus mirabilis + Serratia marcescens	*Enterococcus faecalis + Staphylococcus aureus*	-
Patient 9	*Enterobacter cloacae*	*Escherichia coli*	*Staphylococcus epidermidis*
	Enterobacter cloacae	*Escherichia coli*	*Staphylococcus aureus*
Patient 10	*Escherichia coli + Enterococcus faecalis*	*Enterococcus faecalis*	*Staphylococcus simulans*
	Morganella morganii	*Enterococcus faecalis*	*Klebsiella pneumoniae*
Patient 11	*Enterobacter cloacae*	*Proteus mirabilis + Kebsiella pneumoniae*	*Staphylococcus epidermidis*
	Enterobacter cloacae	*Proteus mirabilis*	*Staphylococcus epidermidis*

[1]Patient with a methylene blue test that was positive for dehiscence.

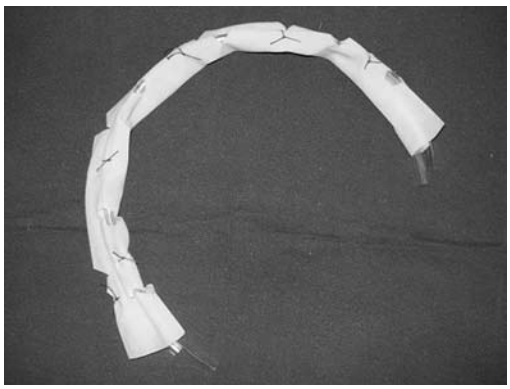

Figure 1 Watterman drain.

latex tubular drain all cultures were positive and the most frequent bacteria were *Enterobacter* spp., *Enterococcus* spp., *Proteus* spp. and *Pseudomonas* spp.; all the cultures of the tubulolaminar Watterman drains were also positive and the most frequent bacteria were *Serratia* spp., *Morganella* spp., *Proteus* spp. and *Enterobacter* spp..

Microbiological evaluation of the drain end located in the peritoneal cavity showed a similar frequency of culture positivity and similar bacterial species identified in the peritoneal fluid for all drains (Table 4).

Subjective evaluation

Drain handling and emptying of the collecting bag were considered easy for all drain types. The tubular latex drain was considered to be the most painful and the silicone channeled drain was considered to present fewer unpleasant odors (Table 5).

Table 5 Subjective evaluation of the ease of handling and comfort of the abdominal drains

	Blake	Watterman	Latex
Ease of emptying the collecting bag			
Very easy	7	7	9
Easy	3	4	2
Difficult	1	0	0
Very difficult	0	0	0
Odor during the dressings			
None	9	1	5
Bad	2	3	3
Very bad	0	7	3
Pain at the drain site (pain scale)			
0 (no pain)	6	5	2
1	2	3	3
2	2	2	0
3	3	1	3
4	0	0	1
5 (very intense pain)	0	0	2
Pain during drain removal (pain scale)			
0 (no pain)	7	4	3
1	2	6	2
2	2	1	1
3	0	0	2
4	0	0	1
5 (very intense pain)	0	0	2

DISCUSSION

Drainage of body cavities has been practiced in medicine for a long time. During the last three decades, surgeons have made efforts to investigate the value of prophylactic drainage after abdominal surgery in controlled randomized clinical trials[8,9]. The utility of closed suction drains after gastrointestinal procedures

has long been debated. Although there is some data against the use of prophylactic drains, bariatric surgeons often use them for a variety of reasons: as an early alert to the presence of leakage and hemorrhage, and as a resource for the treatment of these complications[16].

It was not the subject of this work to study the benefits or disadvantages of the presence of drains or how often they are used. For this type of study, a greater number of patients must be evaluated. Although there is a lack of consensus regarding prophylactic drainage in gastric surgery[9], at our institution, we always use tubular closed drains without suction in gastrointestinal procedures and, in accordance with many bariatric centers, prophylactic drains are routinely used in bariatric surgery. On the other hand, the effectiveness of a tubular closed drain without suction is very low for prolonged postoperative periods and may impair the diagnosis and treatment of fistulae after bariatric surgery, especially those with delayed occurrence[5]. In an experimental study, the tubular drain was found to be obstructed early, 24 to 48 h after its introduction, due to envelopment by the omentum and penetration of omental fringes into the draining orifices. Contamination around the drain has also been observed, causing washing for relief of obstruction to be risky[6].

Early studies have demonstrated that the persistence of serous drainage after obstruction of drains placed in the peritoneal cavity originates from a reaction by the organism to the presence of a foreign body, in this case the drain itself[7]. The migration of bacteria into the peritoneal cavity through the drain has also been reported[6,7].

In general, tubular closed drains tend to result in lower infection rates compared to laminar open catheters. On the other hand, laminar open drains are less frequently obstructed[11]. Thus, it is pertinent to look for an alternative way of keeping drains permeable for a prolonged period of time in order to facilitate the diagnosis and management of fistulae after bariatric surgery, especially those occurring in a delayed manner.

In the present study, the performance of the latex tubular drain without suction was similar to that of the Watterman model, which functions as a tubulolaminar drain, also without suction. There was no difference in terms of drained volume, culture positivity or diversity of the bacterial species isolated. Subjective evaluation revealed that the tubulolaminar drain had an unpleasant odor when dressings were changed compared with the tubular drain, which was more painful when handled.

The silicone channeled closed drain with vacuum and without multiple perforations had some advantages over the two more traditional models, such as a lower incidence of obstruction and pain at the site of insertion, as well as easy handling, and represents a more recent alternative that deserves to be evaluated in view of the additional costs[12].

In the present study, a persistently greater volume of daily drainage was observed with the silicone channeled closed drain, suggesting lower obstruction rates. A lower incidence of pain and fewer unpleasant odors were also recorded. Bacterial contamination by the retrograde route occurred in 81% of cases, however, the bacteria most frequently identified had a less pathogenic profile compared to the other two types of drain.

Thus, we can conclude that the silicone drain with multiple channels has a more prolonged permeability, and is recommended as an alternative for drainage of the peritoneal cavity after bariatric surgery. This recommendation is made in view of the fact that dehiscence can manifest in a delayed manner, as we experienced a patient with staple line dehiscence on the seventh postoperative day.

COMMENTS

Background

With the current increase in bariatric surgery, some complications such as, intraoperative bleeding and dehiscence of anastomoses, although infrequent, are matters of concern. The resources for the early diagnosis of these complications are limited. Drainage of the peritoneal cavity may result in the early identification and treatment of fistulae. Different types of drains are available but the search is ongoing for the ideal model.

Research frontiers

A closed-system model of a silicone drain, with multiple channels in its intra-abdominal portion (Blake®Ethicon) was recently produced. This silicone channeled closed drain with vacuum had some advantages over the two more traditional models, such as a lower incidence of obstruction and pain at the site of insertion, as well as easy handling.

Innovations and breakthroughs

This silicone drain (Blake®Ethicon) is being used by a great number of surgeons around the world and for a wide variety of surgical procedures such as cardiothoracic surgery, transplantation and bariatric surgery

Applications

This study suggests that the silicone drain is a good alternative for drainage of the peritoneal cavity after bariatric surgery, if the surgeon decides to drain it.

Terminology

Bariatric surgery is carried out in severely obese patients with the objective of reducing body weight and the comorbidity related to obesity. Dehiscence is any rupture or opening of surgical sutures. Drains are a device by which a channel or open area may be established for the exit of fluids or purulent material from a cavity, wound, or infected area.

Peer review

The authors compared the performance of different types of abdominal drains used during bariatric surgery. This article is interesting and well written.

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Celecoxib enhances the detoxification of diethylnitrosamine in rat liver cancer

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Abstract

AIM: To study the effect of celecoxib (CXB) on diethylnitrosamine activation through the regulation of cytochrome P450 in a hepatocarcinogenesis model.

METHODS: Six-week-old male Sprague-Dawley rats were randomly divided into five groups, a non-treated group (NT), a diethylnitrosamine-treated group (DEN), a DEN+CXB-treated group (DEN+CXB), and CXB 8 d-treated and CXB 32 d-treated groups. The effects of celecoxib on the enzymatic activities of CYP1A1, 2A, 2B1/2, and 2E1 were assessed in hepatic microsomes 24 h after DEN administration. Changes in CYP1A1 and CYP2B1/2 protein expression were also evaluated. The rate of DEN metabolism was measured by the production of the deethylation metabolite acetaldehyde, and the denitrosation metabolite nitrite.

RESULTS: DEN+CXB administration produced a significant increase in the enzymatic activities of CYP2B1/2 and 1A1, whereas it did not change the activities of CYP2A and 2E1, compared to that of the DEN group. CXB treatment for eight days did not produce a significant effect on enzymatic activity when compared to the NT group; however, when it was administered for prolonged times (CXB 32 d group), the enzymatic activities were increased in a similar pattern to those in the DEN+CXB group. The observed increase in the enzymatic activities in the DEN+CXB group was accompanied by an increase in the CYP2B1/2 protein levels; no changes were observed in the levels of CYP1A1. *In vitro*, CXB increased the denitrosation of DEN, a pathway of metabolic detoxification. The addition of SKF-525A, a preferential inhibitor of CYP2B, abrogated the denitrosation of DEN.

CONCLUSION: These results suggest that the mechanism of action of CXB involves enhancement of the detoxification of DEN by an increasing denitrosation *via* CYP2B1/2.

Key words: Hepatocarcinogenesis; Chemoprevention; Diethylnitrosamine; Denitrosation; Celecoxib; Cytochromes P450

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common tumors, with about one million new cases per

year worldwide. Despite progress in early diagnosis and novel therapies, the overall survival of HCC patients has not been significantly improved over the last three decades. Therefore, preventive strategies are of paramount importance and need to be actively explored in order to reduce the incidence of this disease[1].

Numerous epidemiological studies have demonstrated that long-term use of cyclooxygenase-2 (COX-2) specific inhibitors, such as celecoxib (CXB), are associated with a reduced incidence of several types of cancer[2]. Studies in rodents have shown that CXB inhibits the development of chemically induced cancers, including colon, skin, urinary bladder, and breast[3-6]. The proposed mechanisms for the effects of CXB in these models include inhibition of cell proliferation, reduction of angiogenesis and induction of apoptosis[7]. Recently, we have demonstrated that CXB acts as a chemopreventive agent against the development of preneoplastic lesions induced by diethylnitrosamine (DEN), 2-acetylaminofluorene and partial hepatectomy in the modified resistant hepatocyte (MRH) model[8]. However, the exact mechanism of action by which CXB decreases liver preneoplastic lesions remains unclear, because there was no evidence of apoptosis or of changes in COX-2 expression or PGE2 production after CXB treatment. The observed reduction in proliferation markers was not sufficient to explain the reduced number of preneoplastic lesions, thus other mechanisms must be involved in the CXB effect, probably during the initial stages of hepatocarcinogenesis.

In the MRH model, DEN bioactivation is required to produce preneoplastic lesions and subsequently HCC[9]. The metabolic activation of DEN occurs during the first hours after administration, *via* cytochrome P450 (CYP)-dependent α-hydroxylation, which results in an ethylating agent capable of forming DNA adducts. The CYP1A1/2, 2B, 2A1/2 and 2E1 subfamilies are the major enzymes involved in the bioactivation of DEN[9-11]. In addition to the activation reaction, a denitrosation reaction may also occur, which results in nitrite production. Nitrite formation is an alternative pathway for the formation of an alkylating intermediate, and represents a carcinogen detoxification pathway[12-14]. These two pathways of DEN metabolism could occur in parallel, and although some studies suggest that both pathways are catalyzed by the same CYP enzyme, the participation of distinct isoforms must be considered. In the absence of CYP inducers, the predominant reaction is activation; nevertheless, when specific isoforms are induced, the two mechanisms compete with each other, favoring the DEN detoxification pathway[9,14].

Since there is no information in the literature about CYP regulation by CXB as a chemopreventive mechanism, the aim of this study was to determine the effect of CXB on DEN activation by affecting CYP regulation in the MRH model. These data demonstrate that the preferential modulation of CYP2B1/2 by CXB enhances DEN detoxification, which therefore blocks the initiation of the hepatocarcinogenic process.

MATERIALS AND METHODS

Materials

DEN was purchased from Sigma Chemical Co. (St. Louis, MO). Ethoxy- and pentoxy-resorufin were purchased from Molecular Probes, Inc. (Eugene, OR). Electrophoresis reagents were purchased from Bio-Rad (Hercules, CA). The monoclonal anti-rat CYP1A1 antibody was purchased from Oxford Biochemicals Research, Inc. (Oxford, MI). The monoclonal anti-rat CYP2B1/2 antibody was kindly provided by Dr. Colin Jefcoate (University of Wisconsin-Madison, Dept. of Pharmacology, Madison, WI). The horseradish peroxidase-conjugated goat anti-mouse IgG antibody was acquired from Pierce Protein Research Products (Rockford, IL).

Experimental diet

CXB was extracted from the commercial drug Celebrex® (Pfizer, Mexico City, Mexico). The identity and purity of the molecule was above 99%, as determined by nuclear magnetic resonance analysis in the Department of Chemistry at CINVESTAV (Mexico City, Mexico). Diet 5001 containing 1500 ppm of CXB was prepared by Purina Test Diet (Richmond, IN).

Animals

Six-week-old male Sprague-Dawley rats were purchased from Harlan Industries (Mexico City, Mexico). Rats were fed ad libitum and housed in a controlled environment with a 12 h light/dark cycle, 50% relative humidity and a temperature of 21 ± 2℃. All experiments were performed according to the guidelines established by the Institutional Animal Care Committee in agreement with Mexican Official Norm NOM-062-ZOO-1999.

Experimental procedure

After acclimation, the rats were separated into five treatment groups (Figure 1). In the non-treated (NT) group, rats were fed with 5001 basal diet and sacrificed eight days after the beginning of the experiment; the DEN and DEN+CXB groups received a single intraperitoneal dose of DEN (200 mg/kg) and were sacrificed 24 h later. The DEN group was fed the basal diet. The DEN+CXB group was pretreated with CXB from seven days before DEN administration until sacrifice. The CXB 8 d and CXB 32 d groups were treated only with CXB for the indicated times. Animals were sacrificed by cervical dislocation and the livers were then removed and processed to obtain microsomes, as described by Mayer *et al*[15].

Enzymatic activities

Alkoxyresorufin metabolism assays: Microsomal *O*-dealkylation of 7-ethoxy-(EROD, CYP1A1) and 7-pentoxy-resorufin (PROD, CYP2B1/2) were measured fluorometrically at 37℃ using 530 and 585 nm excitation and emission wavelengths, respectively[16,17].

***p*-Nitrophenol hydroxylase (PNPH) assay:** The

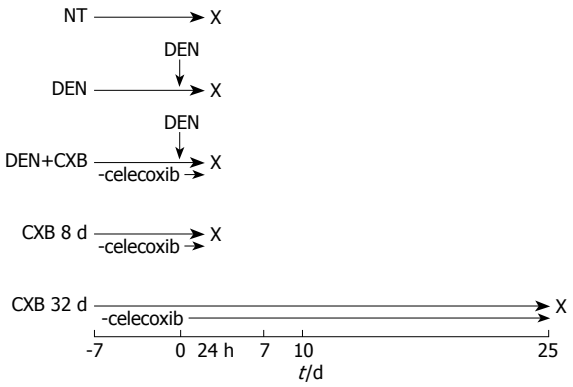

Figure 1 Schematic representation of CXB administration in the hepatocarcinogenesis model. In the NT group, rats were maintained on a basal diet. DEN and DEN+CXB groups were treated with DEN. The CXB diet was given from 7 d before DEN administration until sacrifice, indicated with X ($n = 4$).

activity of CYP2E1 was measured by the formation of 4-nitrocatechol, which was determined spectrophotometrically at 546 nm[18].

7α-Testosterone hydroxylation: The activity of CYP2A1 was determined in microsomal suspensions obtained from treated and control rats as previously described[19]. Protein concentration was determined by Lowry's method[20] using bovine serum albumin as a standard.

Immunoblotting

Microsomal proteins (15 and 30 μg/lane for CYP2B1/2 and CYP1A1, respectively) were separated by 10% SDS-PAGE. Proteins were blotted onto PVDF membranes. These membranes were blocked overnight at 4℃ with 100 mmol/L glycine, 1% BSA and 5% non-fat dry milk in a PBS-1% Triton X-100 solution. Then, membranes were challenged with anti-rat CYP1A1 or 2B1/2 antibodies for 1 h at room temperature, followed by incubation with a horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. The specific protein bands were visualized by chemiluminescence (Santa Cruz Biotechnology, Inc.) and exposure to radiographic film. Densitometric analysis of bands was carried out using Sigma Gel software (Jandel Scientific, San Rafael, CA).

In vitro biotransformation of DEN by rat hepatic microsomes

The rate of DEN metabolism in control and CXB-treated rat hepatic microsomes was measured by the production of both a deethylation metabolite, acetaldehyde, and a denitrosation metabolite, nitrite, as previously described[12,14,21]. The enzymatic assay was performed in a final volume of 1 mL TMP buffer (50 mmol/L Tris-HCl, 10 mmol/L $MgCl_2$, 150 mmol/L KCl, pH 7.0) containing 0.5 mg rat hepatic microsomal protein, 1.2 mmol/L NADPH and 50 mmol/L DEN. Reactions were initiated by adding DEN and incubating at 37℃ for 30 min, and were then stopped by adding 0.1 mL 25% $ZnSO_4$ and 0.1 mL saturated $Ba(OH)_2$ in an ice bath. Samples were vortexed and centrifuged at 5000 *g* for 10 min. One-hundred microliter aliquots of supernatant were used for nitrite measurements using a specific colorimetric assay kit (Cayman Chemical Co., Ann Arbor, MI), according to the manufacturer's instructions. Acetaldehyde production was determined by HPLC in a Waters Liquid Chromatography model 600 using an Xterra C18 phase reverse column (3.9 mm × 150 mm), as previously described[21]. As a control for CYP2B1/2-specificity, these assays were carried out in the presence of 50 mmol/L SKF-525A.

Statistical analysis

Data are presented as mean ± SD. Analysis of variance and the Bonferroni test were used to assess statistical differences among the tested groups, and the level of significance was set at $P < 0.05$. All statistical analyses were performed using SigmaStat software version 3.1 (Systat Software, Inc., Point Richmond, CA).

RESULTS

CXB modulates the enzymatic activity of CYPs

To determine whether the chemopreventive effect of CXB is associated with changes in the enzymatic activities of some CYPs, the activities of CYP1A1, CYP2A1, CYP2B1/2 and CYP2E1 were determined 24 h after DEN administration (Table 1). Eight days of CXB treatment did not produce a significant effect on any of the evaluated enzyme activities. DEN treatment significantly decreased the CYP1A1 and CYP2A1 activities by 66% and 58%, respectively, whereas CYP2B1/2 and CYP2E1 activities were increased 3.6- and 2.5-fold, respectively, in comparison to the NT group. When CXB was administered in combination with DEN (DEN+CXB group), the CYP1A1 activity was increased 3.5-fold and the CYP2B1/2 activity was increased 9-fold over the DEN group. Compared with the NT group, the increase in CYP2B1/2 activity was 33-fold, while no significant changes were observed for CYP1A1 activity. On the other hand, the DEN+CXB treatment had no influence on the activities of CYP2E1 and CYP2A1 (Table 1). Interestingly, the prolonged treatment with CXB (CXB 32 d group) produced an increase in the majority of the enzymatic activities analyzed: CYP1A1, CYP2B1/2 and CYP2E1. These results suggest that pretreatment with CXB in combination with the administration of DEN elicited a preferential induction of CYP1A1 and 2B1/2 enzymatic activities, with the 2B1/2 isoforms induced to a greater degree.

Celecoxib induces the expression of CYP2B1/2 but not CYP1A1

To determine whether the increases observed in the enzymatic activities of CYP1A1 and 2B1/2 were related to increases in protein levels, these isoforms were analyzed by immunoblotting. In the DEN-treated group, there were no significant differences in the protein expression of CYP1A1 and CYP2B1/2 compared to the NT group. The pretreatment with CXB in the DEN+CXB group significantly increased CYP2B1/2 protein expression (2.5-fold), but it had no significant effect on CYP1A1 protein expression compared to the DEN group (Figure 2).

Table 1 Effect of celecoxib on hepatic microsomal enzyme activities in a hepatocarcinogenesis assay

Treatment	Alkoxyresorufin *O*-dealkylation activity (pmol resorufin/min per mg protein)		Testosterone hydroxylase activity (pmol of product/min per mg protein)	*p*-Nitrophenol hydroxylase activity (nmol 4-nitrocatechol/min per mg protein)
	EROD (CYP1A1)	PROD (CYP2B1/2)	7α-OHT (CYP2A1/2)	PNPH (CYP2E1)
NT	13.1 ± 0.5	2.0 ± 0.5	129.2 ± 18.9	0.38 ± 0.07
CXB 8 d	13.3 ± 4.3	4.2 ± 2.2	ND	0.53 ± 0.16
DEN	4.5 ± 1.3[a]	7.3 ± 3.2[a]	54.2 ± 19.2[a]	0.97 ± 0.14[a]
DEN+CXB	15.9 ± 4.8[b]	65.9 ± 35.4[a,b]	66.0 ± 5.9[a]	0.80 ± 0.25[a]
CXB 32 d	54.5 ± 11.3[a]	114.2 ± 23.2[a]	151.5 ± 29.5	1.40 ± 0.44[a]

Male rats were treated with a single dose of DEN (200 mg/kg) *ip* and sacrificed 24 h after administration (DEN and DEN+CXB groups). The CXB diet was provided 7 d before DEN treatment and until sacrifice (8 d). The CXB 8 d and CXB 32 d groups received only a CXB-containing diet. [a]Significantly different from the NT group; [b]From the DEN group, according to the Bonferroni test ($P < 0.05$). Values shown are the mean ± SD from $n = 4$. ND: Not determined.

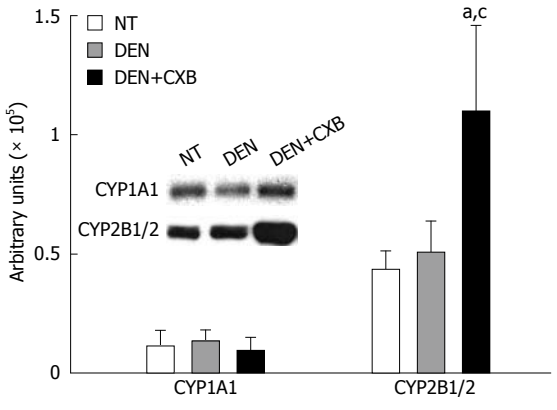

Figure 2 Immunodetection of hepatic microsomal CYP isoforms from DEN- and/or CXB-treated rats. Microsomal proteins (15 and 30 μg/lane for CYP2B1/2 and CYP1A1, respectively) were separated by SDS-PAGE and tested for different CYPs using specific anti-rat CYP antibodies. The bands in the inset box correspond to CYP2B1/2 and CYP1A1 protein detected in the NT, DEN and DEN+CXB groups. The graphic represents the densitometric analysis of the CYP amounts in the experimental groups. [a]$P < 0.05$, *vs* NT group; [c]$P < 0.05$ *vs* DEN group, Bonferroni test. $n = 4$ for all groups.

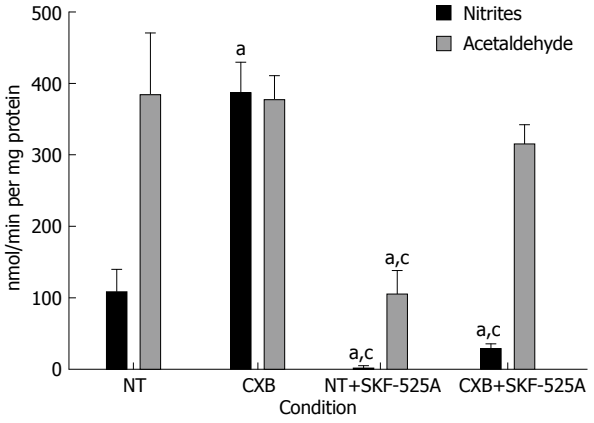

Figure 3 *In vitro* metabolism of DEN in non-treated and CXB-treated rat hepatic microsomes. The denitrosation rate was measured by the production of nitrites, and the deethylation rate was measured by the production of acetaldehyde in the presence or absence of SKF-525A. Values are presented as mean ± SD. [a]$P < 0.05$, *vs* NT group; [c]$P < 0.05$ *vs* CXB group. Bonferroni test, $n = 4$ for all groups.

In summary, these results show that CXB differentially affects these two isoforms; the increase in the protein expression of CYP2B1/2 suggests that the regulation might be at the transcriptional level, while in the case of CYP1A1, CXB seems to regulate only the enzymatic activity.

CXB favors detoxification by denitrosation of DEN

To explore whether regulation of the CYP isoforms by CXB induces the detoxification pathway of DEN as a chemopreventive mechanism, nitrite and acetaldehyde yields were measured in the microsomes of non-treated and CXB-treated rats. We used rat microsomes treated with CXB for 32 d, where the pattern of induction of enzymatic activities was similar to the pattern observed in the DEN+CXB group (with preferential induction of the 2B1/2 isoform), because the eight days of CXB treatment did not produce significant changes in the enzymatic activities.

Microsomes of non-treated rats showed a production of acetaldehyde that was 3.5-fold higher than that of nitrites, which suggests that the predominant route for the DEN metabolism is deethylation, leading to the bioactivation of the carcinogen.

Microsomes obtained from CXB-treated rats showed a 3.6-fold increase in the rate of denitrosation of DEN, while there was no effect on DEN deethylation. This result indicates an induction of the detoxification pathway of the carcinogen. To confirm whether this effect resulted from induction of the enzymatic activity of CYP2B1/2 by CXB, we included SKF-525A in the assay, a CYP inhibitor that acts preferentially on this isoform. Inhibition of CYP2B1/2 resulted in a 98% reduction in nitrite production in the microsomes isolated from non-treated animals, and a 95% reduction in the microsomes of CXB-treated rats, suggesting that CYP2B1/2 was involved in the denitrosation of DEN under basal conditions (NT animals) and CXB-induced conditions. The deethylation rate in non-treated rat microsomes decreased 73%, and no statistically significant changes were observed in the rat microsomes treated with CXB. One possible explanation for this result is that CYP2E1 and 1A1 are minimally affected by SKF-525A and could be responsible for the acetaldehyde production under these conditions (Figure 3).

DISCUSSION

CXB has shown to have anticancer effects in several

experimental models, including the MRH model, where it showed a striking chemopreventive activity by inhibiting liver preneoplastic lesions in rats[8]. Although that study demonstrated a reduction in proliferation markers and in the nuclear translocation of NF-κB, the exact mechanism of action remains unclear[8].

Altered expression of CYP genes is a common feature in hepatic preneoplastic and neoplastic lesions induced by various carcinogens, including DEN[22]. Therefore, DEN metabolism *via* hepatic microsomal CYPs provides molecular targets for chemoprevention. This study demonstrates that the chemopreventive effect of CXB in the modified resistant hepatocyte model is mediated by changes in DEN metabolism *via* CYP regulation. CXB treatment for 8 d did not induce significant changes in enzymatic activities; however, when it was administered for 32 d or in combination with DEN, CXB strongly enhanced the enzymatic activity of CYP2B1/2. Moreover, CXB treatment increased the nitrite levels, which have been proposed to result from the DEN detoxification pathway[12,13]. This finding supports the explanation that the chemopreventive activity of CXB is carried out by reducing carcinogen-induced DNA damage, thus preventing the initiation of hepatocarcinogenesis. We propose that the effect of CXB is due to the preferential induction of CYP2B1/2. This hypothesis is reinforced by *in vitro* results, where the increase in DEN denitrosation elicited by CXB was inhibited by the addition of SKF-525A, an inhibitor of several CYP isoforms including 2B1/2B2, 3A1/2 and 2A, whereas CYP2E1 and 1A are less affected[23]. The deethylation rate was not affected by the inhibitor, suggesting that CYP2E1, and possibly CYP1A, could be the main isoforms involved in this pathway.

According to a previous report, the enzymatic activity of CYP2E1 increased with DEN treatment[24]. This is congruent with the participation of this isoform in DEN metabolism[10,24]. However, CXB did not have any effect on this increase, suggesting that there is no contribution of CYP2E1 to the chemoprotective effect of CXB. On the other hand, CXB reversed the effect of DEN on CYP1A1-specific EROD activity. A decrease in the CYP1A1 enzyme activity in preneoplastic lesions induced by DEN has been previously reported[25]. Induction of CYP1A1 has been related to chemoprevention[26]; thus, the induction of this isoform by CXB could explain its chemopreventive effect, but comparing the levels of enzymatic activity induced reveals that its participation is probably minor compared to CYP2B1/2. Additionally, CYP2A was not affected by CXB, and considering that it is partially affected by SKF-525A, this isoform could be involved in the deethylation reaction of DEN, although to a lesser extent than CYP2E1.

This is the first report that describes the effect of CXB on hepatic CYP regulation. Other chemoprotectors have been shown to act in a similar way. For example, among their multiple effects, diallyl sulfide, indole-3-carbinol, *d*-limonene and bicyclol induced the enzymatic activity of several CYP isoforms, including CYP2B1/2[21,26]. In particular, the effect of bicyclol on CYP2B1 was associated with an increase in the denitrosation rate of DEN[21]. In that case, bicyclol reduced the Km values for denitrosation below the values of deethylation, which may be attributed to the induction of specific CYPs. According to these results on DEN metabolism, the chemopreventive CXB effect in the MRH model could be similar to that of bicyclol[21], mediated mainly by the 2B1/2 isoform. Isoforms of the 1A, 2A, 2B and 2E CYP families share a broader overlap in substrate selectivity. In addition, a single enzyme can bind a variety of substrates, multiple substrates, and/or generate multiple products from a single substrate, which makes it difficult to discriminate between these possibilities in *in vivo* systems[27,28]. Further studies are required to clarify the mechanism by which CXB induces the denitrosation of DEN, and whether this is generated simply by the preferential induction of isoforms or by other effects.

In summary, the modulation of several hepatic CYPs by CXB modifies the bioactivation of DEN, favoring detoxification *via* denitrosation. This pathway may constitute an additional mechanism of action to explain the chemoprotective effects of CXB at the initiation stage in this hepatocarcinogenesis model.

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COMMENTS

Background

Celecoxib, a non-steroidal antiinflammatory drug, is associated with a reduced incidence of several types of cancer, including hepatocellular carcinoma. Study of the mechanism of action has been possible by means of animal models. In the modified resistant hepatocyte model, celecoxib has shown a chemoprotector effect in the development of liver preneoplastic lesions; however, the action mechanism has not been defined completely.

Research frontiers

Diethylnitrosamine bioactivation is a crucial event in the initiation stage of the modified resistant hepatocyte model, a process dependent on hepatic cytochrome P450 (CYP). Therefore, modulation of liver CYP provides molecular targets for chemoprevention. This study demonstrates that the chemopreventive effect of celecoxib is mediated by changes in diethylnitrosamine metabolism *via* CYP regulation.

Innovations and breakthroughs

Recent investigations have demonstrated several mechanisms through which celecoxib exerts its chemoprotector effect. However, this is the first report that describes the capacity of celecoxib to modulate liver CYP expression and explains how the preferential induction of CYP2B1/2 activates the detoxification pathway by increasing nitrite formation. These effects represent an additional mechanism to elucidate the chemopreventive activity of celecoxib.

Applications

This study contributes to the understanding of the mode of action of celecoxib, which may represent a future strategy for therapeutic intervention in the treatment of patients with a high risk of suffering hepatocellular carcinoma.

Terminology

CYP is hepatic microsomal protein involved in the phase I metabolism. Celecoxib is a nonsteroidal anti-inflammatory drug that specifically inhibits cyclooxygenase-2. Diethylnitrosamine is a carcinogen initiator used in the modified resistant hepatocyte model.

Peer review

The authors examined the capability of celecoxib to modulate CYP as part of its chemopreventive mechanism in the modified resistant hepatocyte model. The results suggest that celecoxib favors the diethylnitrosamine detoxification and contribute to clarifying the chemopreventive mechanism in the chemical hepatocarcinogenesis of rat.

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Efficacy of the revised Vienna Classification for diagnosing colorectal epithelial neoplasias

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Abstract

AIM: To prospectively investigate the efficacy of the revised Vienna Classification for diagnosing colorectal epithelial neoplastic lesions in cold biopsy specimens.

METHODS: Patients were selected for inclusion if they had colorectal epithelial lesions that were not considered suitable for direct endoscopic resection. These included colorectal polyps ⩾ 10 mm and lesions suspected of being carcinomas capable of invading the colorectal submucosa or beyond, including strictures, based on the cold biopsies obtained from each lesion prior to resection. We investigated the relationship between diagnoses based on cold biopsy samples using the revised Vienna Classification and resected specimens of the same lesions, and the therapeutic implications of diagnoses made using the revised Vienna Classification. The same cold biopsy specimens were also examined using the Japanese Group Classification guidelines, and compared with the resected specimens of the same lesions for reference.

RESULTS: A total of 179 lesions were identified. The sensitivity, specificity, positive and negative predictive values of the revised Vienna Classification for distinguishing between intramucosal lesions and submucosal invasive carcinomas in cold biopsy specimens was 22.2%, 100%, 100%, and 71.4%, respectively, and for distinguishing between intramucosal lesions and those invading the submucosa or beyond was 59.7%, 100%, 100%, and 37.6%, respectively. The sensitivity, specificity, positive and negative predictive values of the Japanese Group Classification for distinguishing between intramucosal lesions and submucosal invasive carcinomas in cold biopsy specimens was 83.3%, 91.4%, 83.3%, and 91.4%, respectively, and for distinguishing between intramucosal lesions and those invading the submucosa or beyond was 95.1%, 91.4%, 97.9%, and 82.1%, respectively. A total of 137 of 144 carcinomas that had invaded the submucosa or beyond and three high-grade intraepithelial neoplasias were diagnosed as "carcinoma" using the Japanese Group Classification system.

CONCLUSION: The revised Vienna Classification for cold biopsy specimens has high positive predictive value in the diagnosis of colorectal carcinoma invasive to the submucosa or beyond.

Key words: Biopsy; Cancer; Colonoscopy; Colorectal epithelial neoplasia; Revised Vienna Classification

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INTRODUCTION

Considerable discrepancies have been reported between diagnoses of colorectal epithelial neoplastic lesions made by Western and Japanese pathologists from endoscopic cold biopsies and resected specimens of the same

lesions[1,2]. Japanese pathologists have distinguished five groups of lesions within the spectrum of colorectal epithelial neoplasia for cold biopsy specimens [Japanese Group Classification (JGC)], namely: normal or benign changes (inflammation/hyperplasia) without atypia [Group 1 (G1)]; non-neoplastic lesions with atypia resulting from inflammation, hyperplasia or regeneration [Group 2 (G2)]; neoplastic lesions with low-grade atypia, including adenomas with mild or moderate atypia and lesions difficult to diagnose as neoplastic or non-neoplastic [Group 3 (G3)]; neoplastic lesions strongly suspected of carcinoma, including adenomas with severe atypia [Group 4 (G4)]; and definite carcinoma [Group 5 (G5)], irrespective of intramucosal or submucosal invasion[3,4]. This different criterion for the diagnosis of "colorectal carcinoma" may be the reason why there are fewer discrepancies between diagnoses from cold biopsies and resected specimens by Japanese pathologists.

In the clinical setting, cold biopsies are required to facilitate management decisions for large and/or advanced lesions. The therapeutic implications of resecting adenomatous polyps equal to or larger than 10 mm ($\geqslant$ 10 mm) should be considered carefully because these polyps are at risk of becoming submucosal invasive carcinomas[5]. Compared to the endoscopic diagnosis of colorectal polyps including submucosal invasive carcinomas, the endoscopic diagnosis of more advanced colorectal carcinomas rarely presents a problem and can be referred for surgical resection[6]. However, histopathologic confirmation of these lesions from cold biopsy specimens should always be sought. Discrepancies between diagnoses based on cold biopsies and resected specimens of the same lesions are more likely to occur for these large and/or advanced lesions because cold biopsy-based diagnoses are subject to the limitations of superficiality and sampling errors[3]. In contrast, direct endoscopic resection (ER) without prior cold biopsy of small (< 10 mm) colorectal polyps is feasible and histopathologic examination of completely resected lesions enables adequate diagnosis and appropriate treatment, therefore, cold biopsies for small polyps are not mandatory.

Diagnostic discrepancies do not matter to patients if Western and Japanese physicians understand the implications of their respective pathology reports and apply management strategies that are appropriate to the needs of their patients[7]. However, continued attempts to unify Western and Japanese reporting systems are desirable because merging the terminologies of these systems will help codify the advantages of each into a language that is universally understood[8].

To overcome the differences between the conventional Western criteria and the JGC, the Vienna Classification attempted to combine the basic concepts of the conventional Western criteria, which emphasizes that invasion is an indicator of metastatic potential, with the strong points of the JGC, which values consistency between diagnoses of cold biopsy and resected specimens[2,9]. In the revised Vienna Classification (rVC), histopathologic diagnoses are classified into five categories according to neoplastic severity and depth of invasion. This classification also distinguishes between epithelial neoplastic lesions limited to the mucosa and those invading the submucosa[2].

To examine the efficacy of the rVC for diagnosing colorectal polyps $\geqslant$ 10 mm, and colorectal lesions suspected of being carcinomas invasive to the submucosa or beyond, including strictures, we prospectively compared the diagnoses from cold biopsy specimens using the rVC guidelines with the diagnoses from resected specimens of the same lesions using the World Health Organization (WHO) classification[10]. We investigated the value of the rVC system for distinguishing intramucosal lesions from those capable of invading the submucosa or beyond, with special reference to distinguishing between intramucosal lesions and submucosal invasive carcinomas because of the different therapeutic implications among these lesions. In addition, the same cold biopsy specimens were examined using the JGC guidelines and the resulting diagnoses compared to those obtained from the resected specimens of the same lesions, graded according to the WHO classification.

MATERIALS AND METHODS

Patients

In total, 5465 colonoscopies, sigmoidoscopies or proctoscopies were performed prospectively on 3719 patients at the Toho University Ohashi Medical Center, Tokyo, Japan, between January 2001 and December 2003. The study was approved by the Toho University Ohashi Hospital ethics committee. Signed informed consent was obtained from all participating patients. This study was performed in accordance with the Helsinki Declaration.

Inclusion/exclusion criteria

Patients were selected for inclusion in this study if they had colorectal epithelial lesions that were not considered suitable for direct ER. These included colorectal polyps $\geqslant$ 10 mm and lesions suspected of being carcinomas capable of invading the colorectal submucosa or beyond, including strictures, based on the cold biopsies obtained from each lesion prior to resection. The histopathologic diagnosis of each cold biopsy specimen was compared with the final histopathologic diagnosis of each resected lesion. Exclusion criteria included: no epithelial lesions; polyps < 10 mm; polyps $\geqslant$ 10 mm and lesions suspected of being carcinomas invasive to the submucosa or beyond, including strictures, but with no cold biopsy specimens; the inability to compare the histopathologic diagnosis of cold biopsy specimens with the final histopathologic diagnosis of the resected lesion; carcinoid tumors; familial adenomatous polyposis; inflammatory bowel disease; local recurrence after resection for epithelial neoplastic lesions; and the inability to give informed consent.

Endoscopic evaluation

All lesions were diagnosed macroscopically using

conventional colonoscopes (CF-200I, 230I, or 240I; Olympus Co, Ltd, Tokyo, Japan) by endoscopists who had performed more than 500 colonoscopic procedures by direct visualization. If necessary, the lesions were then delineated using 0.1% indigo carmine solution. Polyps and early colorectal carcinomas were classified as Ⅰp (pedunculated type), Ⅰsp (semipedunculated type), Ⅰs (sessile type), Ⅱa (superficial elevated type), Ⅱb (superficial flat type), or Ⅱc (superficial depressed type) according to the criteria outlined by the Japanese Society for Cancer of the Colon and Rectum[4]. Early colorectal carcinoma was defined as carcinoma with invasion limited to the mucosa or submucosa, regardless of the presence or absence of lymph node metastases[1,4]. Lesions that had become invasive carcinomas and had advanced into the muscularis propria or beyond were classified as exophytic/fungating, endophytic/ulcerative, diffusely infiltrative/linitis plastica, or annular according to the WHO classification[10].

Measurements of lesions and tissue sampling

The size of each lesion was estimated *in situ* by using a fully opened standard biopsy forcep (8 mm) (FB-24Q-1; Olympus) adjacent to the lesion, and measured after resection. The cold biopsies were performed using the same forceps (FB-24Q-1; Olympus). The number of cold biopsy specimens and the areas biopsied were dependent on the discretion of each endoscopist; if possible, specimens were obtained from different areas, and included the edges and the center of the lesion.

Treatment modality

Treatment modality was dependent on the size of the lesion, the endoscopic assessment of the depth of invasion and the degree of stricture, and on factors such as the patient's age and morbidity. This was also aided by the histopathologic diagnoses from cold biopsy specimens according to the JGC as routinely practiced.

Histopathologic evaluation

The cold biopsy specimens were fixed with 10% buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin. The only clinical information available to the examining pathologists was that the specimen in question represented a biopsy/biopsies of a colorectal epithelial lesion. All cold biopsy specimen slides were examined independently by two experienced pathologists, and all discrepancies were resolved by a conjoint review of the slides in question. Histopathologic type and grade was evaluated according to the WHO classification[10]. Histopathologic diagnosis of each cold biopsy specimen was made using both the rVC and JGC guidelines[2-4]. If more than one cold biopsy specimen was taken, the most advanced diagnosis was taken as the final diagnosis of the lesion. After resection, tissue samples of the entire lesion were cut from resected specimens that had been fixed in 10% buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin. The histopathologic diagnoses of resected specimens were made for each lesion using the WHO classification[10]. The relationship between the diagnoses of cold biopsy specimens using the rVC and JGC guidelines, and the depth of invasion in resected specimens of the same lesions was investigated.

Statistical analysis

The sensitivity and specificity, and positive and negative predictive values were all calculated with 95% confidence intervals (CI)[11]. The varying proportion of categorical variables between two groups (i.e. intramucosal lesions *versus* submucosal invasive carcinomas, and intramucosal lesions *versus* those invading the submucosa or beyond) was tested by Fisher's exact test. Statistical significance was defined as $P < 0.05$.

RESULTS

Clinicopathologic data

One patient with subserosal invasive transverse colon carcinoma with three cold biopsy specimens was excluded from the analysis because all three specimens showed necrotic tissue only. There were 171 patients (93 men, 78 women; mean age, 66.9 years; range, 33-93) with 179 lesions. A single lesion was found in 165 (96.5%) cases with five (2.9%) and one (0.6%) patients having two or four lesions, respectively. Ten lesions were located in the cecum (5.6%), 34 in the ascending colon (19.0%), 25 in the transverse colon (14.0%), 11 in the descending colon (6.1%), 46 in the sigmoid colon (25.7%), and 53 in the rectum (29.6%). Eight lesions were classified as Ⅰp (4.5%), seven as Ⅰsp (3.9%), 20 as Ⅰs (11.2%), 13 as Ⅱa (7.3%), six as Ⅱa + Ⅱc (3.4%), seven as exophytic/fungating (3.9%), 63 as endophytic/ulcerative (35.2%), and 55 as annular (30.7%). The lesions ranged from 10 to 180 mm in diameter (mean, 46.8 mm). No carcinomas < 10 mm invading the submucosa or beyond were found. Ileocecal resection ($n = 7$), right hemicolectomy ($n = 40$), partial resection of the transverse colon ($n = 6$), left hemicolectomy ($n = 5$), partial resection of the descending colon ($n = 3$), sigmoidectomy ($n = 32$), anterior resection ($n = 37$), abdominoperineal resection ($n = 7$), subtotal colectomy ($n = 4$), Hartmann's procedure ($n = 3$), transsacral resection ($n = 1$), transanal resection ($n = 4$), and ER ($n = 30$) procedures were performed.

Histopathologic diagnoses of cold biopsy specimens from 179 lesions

A total of 404 cold biopsy specimens were obtained from 179 lesions, ranging from one to six specimens per lesion (mean, 2.3). Five inadequate specimens [exudative material (2); granulation tissue (2); necrotic tissue (1)] were excluded; therefore, 399 cold biopsy specimens were included in the analysis. The histopathologic type and grade of each cold biopsy specimen was classified as follows: four non-neoplastic lesions; one indefinite neoplastic lesion; 31 low-grade intraepithelial neoplasias; 55 high-grade intraepithelial neoplasias; 69 well-differentiated adenocarcinomas; 16 moderately differentiated adenocarcinomas; and three poorly differentiated adenocarcinomas.

Table 1 Relationship between the histopathologic diagnoses of cold biopsy specimens using the revised Vienna Classification and the depth of invasion in resected specimens of the same lesions

Invasion depth[1]	The revised Vienna Classification								Total (%)
	C1	C2	C3	C4.1	C4.2	C4.3	C4.4	C5	
Non-N	2	0	0	0	0	0	0	0	2 (1.1)
LGIN	0	0	12	0	0	0	0	0	12 (6.7)
HGIN	0	0	16	2	1	0	2	0	21 (11.7)
Submucosa	0	0	2	1	0	3	8	4	18 (10.1)
MP or beyond	2	1	1	0	6	4	30	82	126 (70.4)
Total (%)	4 (2.2)	1 (0.6)	31 (17.3)		57 (31.8)			86 (48.0)	179 (100)

C: Category; Non-N: Non-neoplastic; LGIN: Low-grade intraepithelial neoplasia; HGIN: High-grade intraepithelial neoplasia; MP: Muscularis propria. [1]The histopathologic diagnoses of resected specimens were made using the World Health Organization classification. The comparison of two groups (intramucosal lesions (i.e. Non-N, LGIN and HGIN) *versus* submucosal invasive carcinomas) tested by Fisher's exact test showed $P = 0.01$. The comparison of two groups (intramucosal lesions *versus* those that had invaded the submucosa or beyond) tested by Fisher's exact test showed $P < 0.001$.

Table 2 Relationship between the histopathologic diagnoses of cold biopsy specimens using the Japanese Group Classification and the depth of invasion in resected specimens of the same lesions

Invasion depth[1]	The Japanese Group Classification					Total (%)
	G1	G2	G3	G4	G5	
Non-N	2	0	0	0	0	2 (1.1)
LGIN	0	0	12	0	0	12 (6.7)
HGIN	0	0	16	2	3	21 (11.7)
Submucosa	0	0	2	1	15	18 (10.1)
MP or beyond	2	1	1	0	122	126 (70.4)
Total (%)	4 (2.2)	1 (0.6)	31 (17.3)	3 (1.7)	140 (78.2)	179 (100)

G: Group. [1]The histopathologic diagnoses of resected specimens were made using the World Health Organization classification. The comparison of two groups [intramucosal lesions (i.e. Non-N, LGIN and HGIN) *versus* submucosal invasive carcinomas] tested by Fisher's exact test showed $P < 0.0001$. The comparison of two groups (intramucosal lesions *versus* those that had invaded the submucosa or beyond) tested by Fisher's exact test showed $P < 0.0001$.

Relationship between the diagnoses of cold biopsy specimens made under the rVC guidelines and the depth of invasion in resected specimens

The histopathologic diagnoses of 399 cold biopsy specimens made using the rVC guidelines were as follows: 51 for C1; one for C2; 50 for C3; four for C4.1; 14 for C4.2; 28 for C4.3; 98 for C4.4; and 153 for C5. The final rVC diagnoses for the 179 lesions included four C1 lesions, one C2 lesion, 31 C3 lesions, 57 C4 lesions, and 86 C5 lesions. Table 1 shows the relationship between the final histopathologic diagnoses of the cold biopsy specimens using the rVC criteria and the depth of invasion in resected specimens of the same lesions. The resected specimens were diagnosed as follows: 35 intramucosal lesions (two non-neoplastic lesions; 12 low-grade intraepithelial neoplasias; 21 high-grade intraepithelial neoplasias); 18 submucosal lesions; and 126 lesions in the muscularis propria or beyond. The sensitivity of the rVC system to distinguish intramucosal lesions from submucosal invasive carcinomas was 22.2% (95% CI, 3.0%-41.4%), with a positive predictive value of 100%. Specificity and negative predictive value were 100% and 71.4% (95% CI, 58.8%-84.1%), respectively. The comparison of two groups (intramucosal lesions *versus* submucosal invasive carcinomas) tested by Fisher's exact test showed $P = 0.01$. The sensitivity of the rVC system to distinguish intramucosal lesions from lesions invasive to the submucosa or beyond was 59.7% (95% CI, 51.7%-67.7%), with a positive predictive value of 100%. Specificity and negative predictive value were 100% and 37.6% (95% CI, 27.7%-47.4%), respectively. The comparison of two groups (intramucosal lesions *versus* those that had invaded the submucosa or beyond) tested by Fisher's exact test showed $P < 0.001$.

Relationship between the diagnoses of cold biopsy specimens made under the JGC guidelines and the depth of invasion in resected specimens

Histopathologic diagnoses of 399 cold biopsy specimens made using the JGC criteria were as follows: 51 specimens in G1; one in G2; 50 in G3; four in G4; and 293 in G5. The final diagnoses for the 179 lesions using the JGC guidelines were as follows: four G1 lesions; one G2 lesion; 31 G3 lesions; three G4 lesions; and 140 G5 lesions. Table 2 shows the relationship between the final histopathologic diagnoses of the cold biopsy specimens using the JGC guidelines and the depth of invasion in resected specimens of the same lesions. The histopathologic diagnoses made for the 179 resected specimens are described in the section above. The sensitivity of the JGC system to distinguish intramucosal lesions from submucosal invasive carcinomas was 83.3% (95% CI, 66.1%-100%), with a positive predictive value of 83.3% (95% CI, 66.1%-100%). Specificity and negative predictive value were 91.4% (95% CI, 82.2%-100%) and 91.4% (95% CI, 82.2%-100%), respectively. The comparison of two groups (intramucosal lesions *versus* submucosal invasive carcinomas) tested by Fisher's exact test showed $P < 0.0001$. The sensitivity of

the JGC system to distinguish intramucosal lesions from lesions invasive to the submucosa or beyond was 95.1% (95% CI, 91.6%-98.7%), with a positive predictive value of 97.9% (95% CI, 95.5%-100%). Specificity and negative predictive value were 91.4% (95% CI, 82.2%-100%) and 82.1% (95% CI, 70.0%-94.1%), respectively. The comparison of two groups (intramucosal lesions *versus* those that had invaded the submucosa or beyond) tested by Fisher's exact test showed $P < 0.0001$. Three high-grade intraepithelial neoplasias and 137 of 144 carcinomas that had invaded the submucosa or beyond were diagnosed as "carcinoma" (G5) under the JGC guidelines.

DISCUSSION

From a therapeutic point of view, the most important histopathologic distinction in cold biopsy specimens taken from colorectal epithelial neoplastic lesions is whether there is evidence of invasion into the submucosa (or beyond). Histopathologic confirmation of lesions using cold biopsy specimens are ideal for predicting the therapeutic implications of colorectal epithelial neoplasia that cannot be treated by direct ER. We have shown that the rVC system had a high positive predictive value (100%) in diagnosing submucosal invasive carcinomas and carcinomas that had invaded the muscularis propria or beyond from cold biopsy specimens. These results may provide both patients and physicians with valuable information that will facilitate management decisions.

In cases of colorectal polyps, Livstone *et al*[12] reported 13 discrepancies (26%) between the diagnoses from single fractional biopsies and the final diagnoses of colonic lesions in 42 patients with 50 colonic polyps (0.8 to 4.5 cm in diameter). Of these discrepancies, four carcinomas invasive to the submucosa or beyond were found; adenomatous epithelium was detected in the fractional biopsies from two cases and normal colonic epithelium in the other two cases[12]. Pugliese *et al*[13] reported that among 53 patients with 59 colorectal polyps ($\geqslant$ 5 mm), seven cases had carcinomas that had invaded the submucosa or beyond, and four of these had been underestimated from the cold biopsy specimens. Gondal *et al*[14] reported that among 442 patients with a total of 532 colorectal adenomas ($\geqslant$ 2 mm) biopsied by flexible sigmoidoscopy and removed by colonoscopy, the assessment of the intraepithelial neoplasia status was changed in 51 adenomas (10%), and 38 (7%) of these had been underestimated from the cold biopsy diagnoses compared with the diagnoses based on polypectomy samples. Of these lesions, 389 (73%) were < 10 mm in diameter. In addition, four carcinomas invading the submucosa or beyond had been underestimated as being low-grade or high-grade intraepithelial neoplasias[14].

These observations suggest that cold biopsy-based diagnoses underestimate histopathologic diagnoses of the resected lesions in some cases of colorectal epithelial neoplastic lesions. In our study, the rVC system underestimated the distinction between intramucosal lesions and submucosal invasive carcinomas in 26.4% (14/53) of lesions. The sensitivity of the rVC system for distinguishing between intramucosal lesions and submucosal invasive carcinomas was poor (22.2%). Therefore, histopathologic examination of completely resected lesions was essential for the adequate diagnosis and appropriate treatment of the colorectal polyps including submucosal invasive carcinomas[15].

Overall, the rVC system had a high specificity (100%) for the histopathologic diagnoses of carcinomas invasive to the colorectal submucosa or beyond, whereas the sensitivity was poor (59.7%). The rVC system underestimated the distinction between intramucosal lesions and lesions that invaded the submucosa or beyond in 32.4% (58/179) of lesions. The poor sensitivity and high underestimation rate of the rVC system was caused by the high prevalence (80.4%) of submucosal or beyond invasive colorectal carcinomas in our cohort, and because the pathologists used invasion of the submucosa or beyond as an obligatory criterion for the diagnosis of carcinoma.

Direct ER without prior cold biopsy of small (< 10 mm) lesions is usually feasible and histopathologic examination of completely resected lesions enables adequate diagnosis and appropriate treatment. Therefore, cold biopsies for small lesions are not needed and our cases did not include these lesions. Under the JGC criteria, 137 of 144 carcinomas that invaded the submucosa or beyond were diagnosed as "carcinoma" (i.e. G5). The diagnostic criteria for colorectal carcinoma according to the JGC guidelines appear to attach more importance on nuclear features and glandular structures, and the presence of evident invasion into the submucosal layer is not considered mandatory[1]. Therefore, although the cold biopsy forceps were usually capable of sampling intramucosal lesions only, the diagnosis of "carcinoma" was possible under the JGC guidelines. For the same reason, distinguishing between intramucosal lesions and those invasive to the submucosa or beyond, or overestimating intramucosal lesions as those invasive to the submucosa or beyond was not a problem under the rVC guidelines, whereas three high-grade intraepithelial neoplasias were diagnosed as "carcinomas" using the JGC system.

Lesions can be diagnosed as low-grade dysplasia in the West and as carcinomas in Japan due to the differences in interpreting nuclear and structural features[1]. Japanese pathologists consider these features as clues for the diagnosis of carcinoma, but Western pathologists either do not take these features into consideration (such as rounded nuclei and variable shape of glands) or do not attach similar importance to these features with regard to the severity of dysplasia (such as marked hyperchromatism of nuclei and enlarged prominent nucleoli)[1]. These different histopathologic interpretations of the nuclear and structural features of lesions between Western and Japanese pathologists require further investigation.

The use of the rVC guidelines for cold biopsy specimens has a high positive predictive value in diagnosing carcinomas invasive to the colorectal

submucosa or beyond. However, it is of limited value in predicting the depth of invasion assigned to the resected specimens, especially for the diagnosis of submucosal invasive carcinomas. This should be supplemented by endoscopic assessment of the depth of invasion.

COMMENTS

Background

Large differences have been found between Western and Japanese pathologists in their diagnosis of colorectal epithelial neoplastic lesions. To overcome the differences between the conventional Western and the Japanese criteria, the Vienna Classification attempted to combine the basic concepts of the conventional Western criteria, which emphasizes that invasion is an indicator of metastatic potential, with the strong points of the Japanese criteria, which values consistency between diagnoses from cold biopsies and resected specimens. In the revised Vienna Classification, histopathologic diagnoses are classified into five categories according to neoplastic severity and depth of invasion. However, the efficacy of the revised Vienna Classification for diagnosing colorectal epithelial neoplastic lesions has not been reported.

Research frontiers

Diagnostic discrepancies do not matter to patients if Western and Japanese physicians understand the implications of their respective pathology reports and apply management strategies that are appropriate to the needs of their patients. However, continued attempts to unify Western and Japanese reporting systems are desirable because merging the terminologies of these systems will help codify the advantages of each into a language that is universally understood.

Innovations and breakthroughs

This is the first report investigating the efficacy of the revised Vienna Classification for diagnosing colorectal epithelial neoplastic lesions in cold biopsy specimens.

Applications

The revised Vienna Classification of colorectal epithelial neoplastic lesions seeks to be more closely in tune patient management, however, it should be emphasized that cold biopsy-based diagnoses are subject to the limitations of superficiality and sampling errors.

Peer review

The authors prospectively investigated the efficacy of the revised Vienna Classification for diagnosing colorectal epithelial neoplastic lesions in cold biopsy specimens. The studies are well done, and the manuscript is well written.

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Sclerosing cholangitis associated with autoimmune pancreatitis differs from primary sclerosing cholangitis

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Abstract

AIM: To clarify the characteristic features of biliary lesions in patients with autoimmune pancreatitis (AIP) and compare them with those of primary sclerosing cholangitis (PSC).

METHODS: The clinicopathological characteristics of 34 patients with sclerosing cholangitis (SC) associated with AIP were compared with those of 4 patients with PSC.

RESULTS: SC with AIP occurred predominantly in elderly men. Obstructive jaundice was the most frequent initial symptom in SC with AIP. Only SC patients with AIP had elevated serum IgG4 levels, and sclerosing diseases were more frequent in these patients. SC patients with AIP responded well to steroid therapy. Segmental stenosis of the lower bile duct was observed only in SC patients with AIP, but a beaded and pruned-tree appearance was detected only in PSC patients. Dense infiltration of IgG4-positive plasma cells was detected in the bile duct wall and the periportal area, as well as in the pancreas, of SC patients with AIP.

CONCLUSION: SC with AIP is distinctly different from PSC. The two diseases can be discriminated based on cholangiopancreatographic findings and serum IgG4 levels.

Key words: Autoimmune pancreatitis; IgG4; Primary sclerosing cholangitis; Sclerosing cholangitis

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INTRODUCTION

Autoimmune pancreatitis (AIP) is a unique form of pancreatitis in which autoimmune mechanisms are suspected of being involved in the pathogenesis. AIP has many clinical, radiological, serological and histopathological characteristics: (1) elderly male preponderance; (2) initial symptom is frequently painless obstructive jaundice; (3) occasional association with impaired pancreatic endocrine or exocrine function, and various extrapancreatic lesions; (4) favorable response to steroid therapy; (5) radiological findings of irregular narrowing of the main pancreatic duct and enlargement of the pancreas; (6) serological findings of elevated serum γ globulin, IgG, or IgG4 levels, along with the presence of some autoantibodies; and (7) histopathological findings of dense infiltration of T lymphocytes and IgG4-positive plasma cells with fibrosis and obliterative phlebitis in the pancreas[1-3]. Bile duct stenosis occurs frequently with AIP, and the major initial symptom in AIP patients is obstructive jaundice. The lower portion of the common bile duct is frequently stenotic. However, when AIP patients develop stenosis in the intrahepatic bile duct, the cholangiographic appearance is similar to that of primary sclerosing cholangitis (PSC)[4,5]. PSC is a progressive disease involving the intra- and extra-hepatic bile ducts. Despite therapy, PSC sometimes leads to liver cirrhosis. However, since AIP patients respond well to steroid therapy, it is necessary to discriminate between sclerosing cholangitis (SC) associated with AIP and PSC. This study aimed

to clarify the characteristic features of biliary lesions in AIP patients and compare them with those of PSC.

MATERIALS AND METHODS

Study patients

Over a 27-year-period, 43 patients (36 male and 7 female, average age 66.4 years) at Tokyo Metropolitan Komagome Hospital were diagnosed with AIP based on the following clinicopathological criteria: irregular narrowing of the main pancreatic duct on endoscopic retrograde pancreatography (n = 43), pancreatic enlargement on ultrasonography (US) or computed tomography (CT) (n = 42), presence of autoantibodies (n = 22), elevated serum IgG4 level in excess of 135 mg/dL (n = 31), characteristic histological findings in the pancreas (n = 12), and responsiveness to steroid therapy (n = 32). In the 43 AIP patients, 34 had SC (lower bile duct in 34, and intrahepatic bile duct in 4). During the same time, 4 patients were diagnosed with PSC according to appropriate criteria[6].

Methods

The stenotic portion of the bile duct was examined by endoscopic retrograde cholangiopancreatography and/or magnetic resonance cholangiopancreatography, and wall thickening of the bile duct in which stenosis was not obvious on cholangiography was assessed on CT and US. Two experienced gastroenterologists retrospectively reviewed these imaging findings without information on the patients. Extrapancreatic lesions, including sclerosing sialadenitis, sclerosing cholecystitis, and retroperitoneal fibrosis, were evaluated on physical examination, CT, and US. Serum IgG4 levels were measured in 30 AIP patients and 2 PSC patients. Histological examination and immunostaining with anti-IgG4 antibody were performed on specimens of the extrahepatic bile duct (6 AIP patients and 1 PSC patient) and liver (3 AIP and 2 PSC patients).

Statistical analysis

Statistical differences between the two groups were analyzed first by the Kruskal-Wallis H-test, followed by Mann-Whitney's U-test if significant. Other analyses were performed using Fisher's exact test. In all tests, $P < 0.05$ was considered statistically significant.

RESULTS

Clinical features

Men were significantly more commonly affected by SC with AIP than by PSC. Patients' age at diagnosis was significantly older in those with SC with AIP. Among the initial symptoms, obstructive jaundice was the most frequently observed in SC with AIP. Elevated serum IgG4 levels were frequent in SC patients with AIP, but not in the 2 PSC patients examined. Sclerosing diseases were frequently associated with SC with AIP. Ulcerative colitis was present in only 2 young PSC patients (Table 1). Thirty-two SC patients with AIP were treated with steroid therapy, and all of them showed a good response. All PSC patients were treated with ursodeoxycholic acid, and 1 patient underwent steroid therapy for associated ulcerative colitis. Cholangiographic findings progressed gradually in three PSC patients, and one patient ultimately required liver transplantation. All SC patients with AIP had a favorable outcome without liver failure.

Table 1 Clinical differences between sclerosing cholangitis with autoimmune pancreatitis and primary sclerosing cholangitis

	SC with AIP	PSC	P value
Average age (yr)	63.8	39.2	< 0.01
Male/Female	29/5	1/3	< 0.05
Obstructive jaundice +/-	30/4	0/4	< 0.01
Elevated serum IgG4 +/-	26/30	0/2	
Associated sclerosing disease +/-	20/14	0/4	< 0.05
Associated ulcerative colitis +/-	0/34	2/2	< 0.01

SC with AIP: Sclerosing cholangitis with autoimmune pancreatitis; PSC: Primary sclerosing cholangitis.

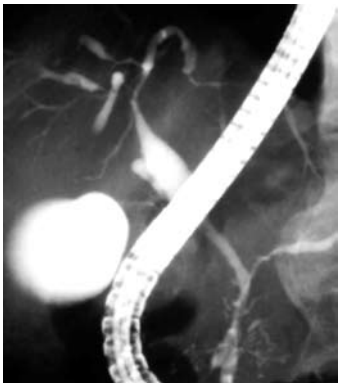

Figure 1 Endoscopic retrograde cholangiography of a patient with autoimmune pancreatitis showing a relatively long stricture of the hepatic hilar bile duct.

Cholangiopancreatographic findings

On pancreatography, narrowing of the main pancreatic duct was detected in all SC patients with AIP, but no abnormal findings were detected in any of the PSC patients. On cholangiography, the intrahepatic bile duct was involved in all PSC patients, but was involved in only four SC patients with AIP (Figure 1). Segmental stenosis of the lower bile duct was observed in all SC patients with AIP, but was not detected in any of the PSC patients. Extensive involvement of the bile duct, showing widespread wall thickening of the middle and upper bile duct where stenosis was not obvious on cholangiography, was detected only in 14 SC patients with AIP, although there was no significant difference between the two groups. A diffusely distributed, beaded and pruned-tree appearance and diverticular formation were detected only in PSC patients (Figure 2 and Table 2). A long stricture was detected in the hepatic hilar region in all 4 SC patients with AIP involving the intrahepatic bile duct.

Histological and immunohistochemical findings

In PSC, the hilar bile duct displayed diffuse fibrosis with moderate lymphoplasmacytic infiltration. The liver of PSC patients showed features of biliary cirrhosis, and fibro-obliterative lesions characterized by onion skin-like periductal fibrosis with predominantly lymphocytic infiltration were observed around the intrahepatic bile

Table 2 Cholangiopancreatographic differences between sclerosing cholangitis with autoimmune pancreatitis and primary sclerosing cholangitis

	SC with AIP	PSC	*P* value
Narrowing of the main pancreatic duct +/-	34/0	0/4	< 0.01
Stenosis of the intrahepatic bile duct +/-	4/30	4/0	< 0.01
Stenosis of the lower bile duct +/-	34/0	0/4	< 0.01
Extensive bile duct wall thickening	14/20	0/4	NS
Beaded appearance	0/34	2/2	< 0.01
Pruned-tree appearance	0/34	3/1	< 0.01
Diverticular formation	0/34	2/2	< 0.01

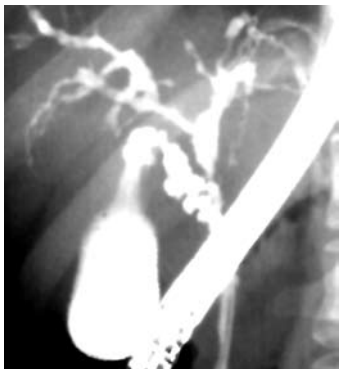

Figure 2 Endoscopic retrograde cholangiography of a patient with primary sclerosing cholangitis showing beaded and pruned-tree appearance.

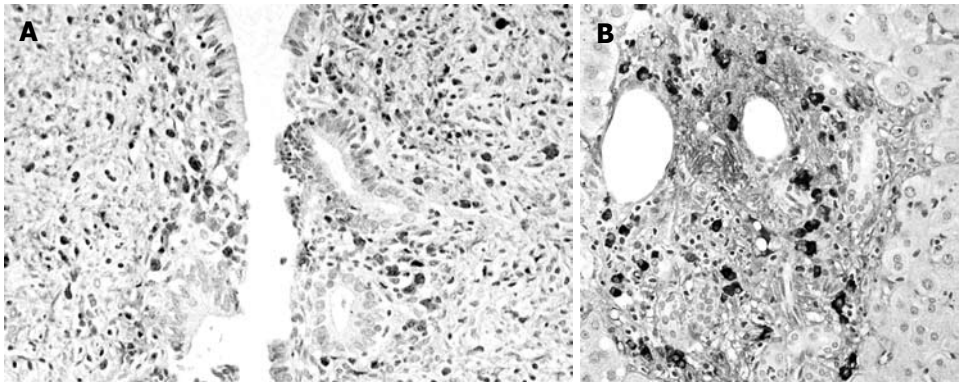

Figure 3 IgG4-immunostaining of the bile duct (A) and liver (B) of a patient with autoimmune pancreatitis. Dense infiltration of IgG4-positive plasma cells was detected in the bile duct wall (A) and the periportal area of the liver (B).

duct. However, infiltration of IgG4-positive plasma cells was not detected in the bile duct or liver.

The histological findings of SC associated with AIP included transmural fibrosis and dense lymphoplasmacytic infiltration of the bile duct wall, along with lymphoplasmacytic infiltration and fibrosis in the periportal area of the liver. Compared with PSC, lymphoplasmacytic infiltration was denser, the degree of fibrosis was less severe, and the onion skin-like appearance was not observed. Dense infiltration of IgG4-positive plasma cells was detected in the bile duct wall (Figure 3A) and the periportal area (Figure 3B), as well as in the pancreas, of patients with AIP.

DISCUSSION

SC is a heterogeneous disease that may be associated with choledocholithiasis, biliary tumor, or infection. SC of unknown origin is called PSC. PSC is progressive despite conservative therapy and involves the intra- and extrahepatic bile ducts, resulting in liver cirrhosis. The effect of steroid therapy is questionable, and liver transplantation currently provides the greatest hope for a possible cure. It occurs among patients in their 30 and 40 s and is frequently associated with inflammatory bowel disease[7,8]. Pancreatograms are not abnormal in most cases[9].

However, an analysis of 192 PSC patients in Japan found that their characteristics differed from those in Western counties, with regard to age distribution and the incidence of complications[10]. In that analysis, the patients were predominantly men, and two peaks in age distribution at diagnosis (20-30 years and 50-70 years) were identified. Compared to younger patients, those aged 40 years or older displayed a lower incidence of associated ulcerative colitis, whereas the incidence of chronic pancreatitis was higher.

SC is frequently associated with AIP, and occurs predominantly in elderly men. The major initial symptom of SC with AIP is obstructive jaundice, which differs from PSC. The most prominent feature on cholangiography for SC with AIP was stenosis of the lower bile duct. When stenosis is found in the intrahepatic or the hilar hepatic bile duct, the cholangiographic appearance is very similar to that of PSC[4,5]. However, a long stricture was detected in the hepatic hilar region in SC patients with AIP, instead of the beaded and pruned-tree appearance that is frequently observed in PSC. Widespread wall thickening of the middle and upper bile ducts was also detected only in SC patients with AIP.

SC patients with AIP responded dramatically well to steroid therapy and showed a favorable outcome[5,9,11]. Histologically, dense infiltration of IgG4-positive plasma cells was detected in the bile duct wall and the periportal area of SC patients with AIP, but it was not detected in PSC patients. SC with AIP is sometimes associated with sclerosing diseases such as sclerosing sialadenitis, sclerosing cholecystitis, or retroperitoneal fibrosis, and these salivary, gallbladder, and retroperitoneal lesions show similar histological findings to those in the bile duct and pancreas. Furthermore, abundant infiltration of IgG4-positive plasma cells is detected in various organs of AIP patients[12,13]. Therefore, we proposed a new clinicopathological entity, an IgG4-related sclerosing disease, which is histopathologically characterized by extensive IgG4-positive plasma cell and T lymphocyte infiltration of various organs. We also suspect that AIP and SC with AIP is a pancreatic and bile duct lesion of this systemic disease[13-15]. Based on the above findings, SC with AIP should be differentiated from

PSC. In particular, since SC with AIP responds well to steroid therapy, discrimination between the two diseases is necessary before making therapeutic decisions. Clinically, serum IgG4 levels and cholangiopancreatographic findings are useful in differentiating between the two diseases.

Considering the predominance of elderly men, the infrequent association with inflammatory bowel disease, and the frequent association with chronic pancreatitis, many older patients diagnosed with PSC in Japan may actually have SC with AIP.

In conclusion, since SC with AIP is induced by different mechanisms to those in PSC, the condition should be differentiated from PSC. The two diseases can be discriminated based on their cholangiopancreatographic findings and serum IgG4 levels.

COMMENTS

Background

When patients with autoimmune pancreatitis (AIP) develop stenosis in the intrahepatic bile duct, the cholangiographic appearance is similar to that of primary sclerosing cholangitis (PSC). PSC is a progressive disease involving the intra- and extrahepatic bile ducts.

Innovations and breakthroughs

Sclerosing cholangitis with AIP is distinctly different from PSC. Only SC patients with AIP had elevated serum IgG4 levels and responded well to steroid therapy. Segmental stenosis of the lower bile duct was observed only in SC patients with AIP, but a beaded and pruned-tree appearance was detected only in PSC patients. Dense infiltration of IgG4-positive plasma cells was detected in the bile duct wall and the periportal area of SC patients with AIP.

Applications

Sclerosing cholangitis with AIP responds well to steroid therapy. The differential diagnosis between sclerosing cholangitis with AIP and PSC is important to ensure optimal patient treatment.

Peer review

The authors described the characteristic features of biliary lesions in autoimmune pancreatitis patients. The paper is well presented and the result are interesting.

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Endoscopic ultrasonography does not differentiate neoplastic from non-neoplastic small gallbladder polyps

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Abstract

AIM: To assess the ability of endoscopic ultrasonography (EUS) to differentiate neoplastic from non-neoplastic polypoid lesions of the gallbladder (PLGs).

METHODS: The uses of EUS and transabdominal ultrasonography (US) were retrospectively analyzed in 94 surgical cases of gallbladder polyps less than 20 mm in diameter.

RESULTS: The prevalence of neoplastic lesions with a diameter of 5-10 mm was 17.2% (10/58); 11-15 mm, 15.4% (4/26), and 16-20 mm, 50% (5/10). The overall diagnostic accuracies of EUS and US for small PLGs were 80.9% and 63.9% ($P < 0.05$), respectively. EUS correctly distinguished 12 (63.2%) of 19 neoplastic PLGs but was less accurate for polyps less than 1.0 cm (4/10, 40%) than for polyps greater than 1.0 cm (8/9, 88.9%) ($P = 0.02$).

CONCLUSION: Although EUS was more accurate than US, its accuracy for differentiating neoplastic from non-neoplastic PLGs less than 1.0 cm was low. Thus, EUS alone is not sufficient for determining a treatment strategy for PLGs of less than 1.0 cm.

Key words: Endoscopic ultrasonography; Neoplastic lesion; Polypoid gallbladder lesion

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INTRODUCTION

Polypoid lesions of the gallbladder (GB) are increasingly detected by ultrasonography (US). Indeed, 4%-7% of healthy individuals have been reported to have polyps of the GB[1,2]. The significance of these polypoid lesions is poorly understood, and the appropriate management of these lesions is controversial. Although most GB polyps are benign, some early carcinomas of the GB share the same appearance as benign polyps. Currently, GB polyps larger than 1 cm should be surgically removed because of the increased risk of malignancy[3]. On the other hand, patients with smaller polyps usually require repeated US and follow-up. Distinguishing between non-neoplastic, neoplastic, and potentially malignant lesions is a major diagnostic dilemma, and the therapeutic options for these lesions remain controversial.

Endoscopic ultrasonography (EUS) is considered to be superior to conventional US for imaging GB lesions, because EUS can provide high-resolution images of small lesions with higher ultrasound frequencies (7.5-12 MHz *vs* 3.5-5 MHz)[4,5]. The improved accuracy of EUS in imaging small GB lesions has been previously reported in a surgical series[5-7]. However, polyps with a maximum diameter of less than 10-15 mm are difficult to differentially diagnose in many cases. The present study assesses the predictive value of EUS in the differential diagnosis of small polypoid lesions (maximum diameter, $\leqslant$ 20 mm) of the GB in a surgical series.

MATERIALS AND METHODS

Patients

Between 1996 and 2006, 365 patients underwent EUS for small (maximum diameter, $\leqslant$ 20 mm) polypoid lesions

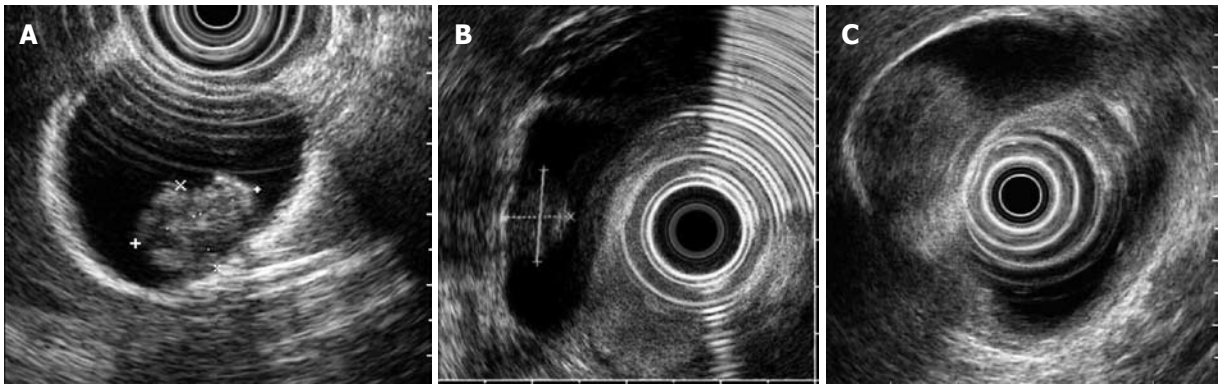

Figure 1 Polypoid lesions of gallbladder. A: Cholesterol polyp of the gallbladder. EUS shows a 13-mm-diameter, granular-surfaced, pedunculated mass with an internal echo pattern characterized by an aggregation of echogenic spots. Histological examination of the surgical specimen showed a cholesterol polyp; B: Adenoma of the gallbladder. EUS shows a 10-mm-diameter, homogeneously isoechoic, pedunculated mass. The histological diagnosis was tubulovillous adenoma with focal high-grade dysplasia; C: Adenocarcinoma of the gallbladder. EUS shows a 19-mm-diameter, smooth-surfaced, heterogeneously echogenic, pedunculated mass. Histological examination of the surgical specimen showed adenocarcinoma.

of the GB detected by transabdominal US. Of these 365 patients, 94 patients who underwent laparoscopic cholecystectomy for GB polyps were enrolled. US was performed as an abdominal screening test for asymptomatic patients or as a detailed examination for patients suspected of having a gastrointestinal disorder because of clinical symptoms. When US revealed polypoid lesions inside the GB, the patient then underwent EUS. In principle, EUS was indicated for polypoid lesions exceeding 5 mm or for potentially neoplastic polyps. Patients with localized adenomyomatosis or diffuse wall-thickening lesions resulting from inflammation were excluded from the study.

Methods

All patients with suspected neoplastic lesions based on EUS underwent surgery. Generally, surgery was not indicated for patients with a EUS diagnosis of non-neoplastic lesions, except for symptomatic cases or patients undergoing combined operations for other abdominal diseases. In our surgical series, the EUS diagnosis was compared with the histopathological diagnosis. Based on the pathological evaluation of specimens obtained upon cholecystectomy, the GB polyps were assigned into two groups: neoplastic (adenoma and carcinoma) and non-neoplastic (cholesterol, inflammatory, and fibrous). In patients with multiple polyps, the size of the largest polyp was measured.

Demographics and EUS findings were prospectively collected at the time of the procedure and were analyzed retrospectively. EUS was performed by one of the authors with knowledge of the ultrasonographic findings. In all cases, the differential diagnosis of polypoid lesions of the GB by EUS and US was made according to the criteria outlined below[5,6].

Cholesterol polyps (Figure 1A) are pedunculated lesions with a granular surface. The internal echo is hyperechoic to isoechoic with a tiny, spotty echo pattern. Relatively large polyps, those greater than 10 mm in diameter, may not give the typical image but may have a spotty echo area. Localized adenomyomatosis is imaged as a sessile echogenic mass containing multiple microcysts or with a comet tail artifact. Neoplastic polyps (adenoma, Figure 1B or carcinoma, Figure 1C) are pedunculated or sessile masses without echogenic spots, multiple microcysts, or comet tail artifacts; the internal echo is hypoechoic to isoechoic and almost homogeneous.

Transabdominal US was performed using a real-time scanner with a 3.5-MHz curved array transducer (SSD-2000; Aloka, Tokyo, Japan). EUS was performed using an echoendoscope with a 7.5-MHz or 12-MHz radial sector scan transducer (GF-UM2, UM3, UM20; Olympus Co., Tokyo, Japan). The GB was visualized from the duodenum and gastric antrum. For sedation, 5 mg of midazolam were administered intravenously.

Statistical analysis

The results were analyzed by Fisher's exact probability test or the Wilcoxon test, as appropriate. Differences were considered significant at $P < 0.05$.

RESULTS

Patient characteristics

Of the 94 patients, 19 had neoplastic lesions and 75 had non-neoplastic lesions. The mean age of the patients with non-neoplastic polyps was 50 ± 12.5 years, and that of patients with neoplastic polyps was 51 ± 11.3 years. Most of the non-neoplastic polyps were cholesterol polyps (56/75, 74.7%). Seventeen polypoid lesions were adenomyomatosis, and two polyps were inflammatory polyps. Adenocarcinoma was found in two patients; and adenomas, in 17. Two of the 17 adenomas contained focal high-grade dysplasia. The prevalence of neoplastic lesions with a diameter of 5-10 mm was 17.2% (10/58); 11-15 mm, 15.4% (4/26), and 16-20 mm, 50% (5/10) (Table 1). The average size of non-neoplastic polyps was 9.8 ± 2.8 mm (5-18). Among neoplastic polyps, the average size of an adenoma without high grade dysplasia, adenoma with high grade dysplasia, and adenocarcinoma were 9.9 ± 3.6 mm (6-17), 12.0 mm (7 and 17), and 19.0 mm (13 and 25), respectively. The average size

Table 1 Histological diagnosis and size of polypoid gallbladder lesions in the surgical series (*n*)

Size (mm)	Cholesterol	Adenomyomatosis	Inflammatory	Adenoma	Cancer	Total
5-10	39	9	0	10	0	58
11-15	14	7	1	3	1	26
16-20	3	1	1	4	1	10

Table 2 EUS and US diagnosis of polypoid gallbladder lesions in the surgical series

	Pathologic diagnosis (*n*)			
	Cholesterol polyp	Adenomyomatosis	Inflammatory polyp	Neoplastic lesions
EUS diagnosis				
Cholesterol	47	4	0	7
Adenomayomatosis	0	11	1	0
Neoplastic lesion	9	2	1	12
US diagnosis				
Cholesterol	41	8	0	10
Adenomayomatosis	0	4	1	0
Neoplastic lesion	15	5	1	9

Table 3 EUS and US diagnosis according to the size of the polypoid gallbladder lesion

	Pathology (*n*)					
	Cholesterol		Adenomyomatosis		Neoplastic lesion	
Size (mm)	5-10	11-20	5-10	11-20	5-10	11-20
EUS						
Cholesterol	33	14	0	0	6	3
Adenomyomatosis	6	0	4	7	1	1
Inflammatory	0	0	0	1	0	1
Neoplastic lesions	4	1	0	0	4	8
US						
Cholesterol	32	9	0	0	7	8
Adenomyomatosis	7	1	1	3	1	4
Inflammatory	0	0	0	1	0	1
Neoplastic lesions	8	2	0	0	2	7

Table 4 Differential diagnosis between neoplastic and benign polyps by EUS and US

	Diagnosis by postoperative histological examination	
	Neoplastic polyp	Non-neoplastic polyp
Diagnosis by EUS		
Neoplastic polyps	12	12
Non-neoplastic polyps	6	64
Diagnosis by US		
Neoplastic polyps	9	21
Non-neoplastic polyps	10	54

of neoplastic polyps including adenoma with high grade dysplasia and carcinoma tended to be larger than neoplastic polyps without high grade dysplasia and non-neoplastic polyps.

Differential diagnosis by EUS and US

Differential diagnosis by EUS and US was successful in 70 (74.5%) and 54 (57.4%) of 94 patients, respectively; the difference between these rates was significant (P = 0.014). When the results of EUS were assessed according to the pathological results (Table 2), cholesterol polyps were correctly identified in 47 of 56 patients (83.9%). The unsuccessful diagnoses included nine cases that were misjudged as adenoma. Adenomyomatosis was correctly identified in 11 of 17 patients (64.7%). Among the six misdiagnosed cases, four were cholesterol polyps and two were neoplastic polyps. Neoplastic polyps were correctly identified in 12 of 19 patients (63.2%). The unsuccessful diagnoses included seven cases misjudged as cholesterol polyps. Five of the seven cases were less than 1.0 cm in size, and another of the cases was 17 mm in size before surgery. EUS showed a homogeneously isoechoic, pedunculated mass, and abdominal CT showed an enhanced polypoid mass of the GB in the arterial phase. Therefore, we diagnosed it as an early cancer. However, this polyp was confirmed to be a cholesterol polyp after cholecystectomy (Figure 2). Of the 19 neoplastic polyps, two were adenocarcinoma, with diameters of 10 and 19 mm, respectively.

Table 3 shows the EUS results categorized according to the size of the polypoid GB lesion (< 10 mm *vs* 10-20 mm). Of the 58 cases with a diameter less than 10 mm, EUS correctly distinguished 84.6% (33/39) of the cholesterol polyps, 36.4% (4/11) of the adenomyomatosis, and 50.0% (4/8) of the neoplastic lesions. Of the 36

Table 5 Sensitivity, specificity, and accuracy of EUS and US diagnoses for neoplastic lesions according to the size of the polypoid gallbladder lesion (%)

	Sensitivity	Specificity	PPV	NPV	Accuracy
EUS					
Overall	66.7	84.2	50.0	91.4	80.9
5-10 mm	44.4	86.0	36.4	89.6	79.7
11-20 mm	88.9	81.5	61.5	95.7	83.3
US					
Overall	47.4	72.0	30.0	84.4	67.0
5-10 mm	20.0	83.3	20.0	83.3	72.4
11-20 mm	77.8	51.9	35.0	87.5	63.9

PPV: Positive predictive value; NPV: Negative predictive value.

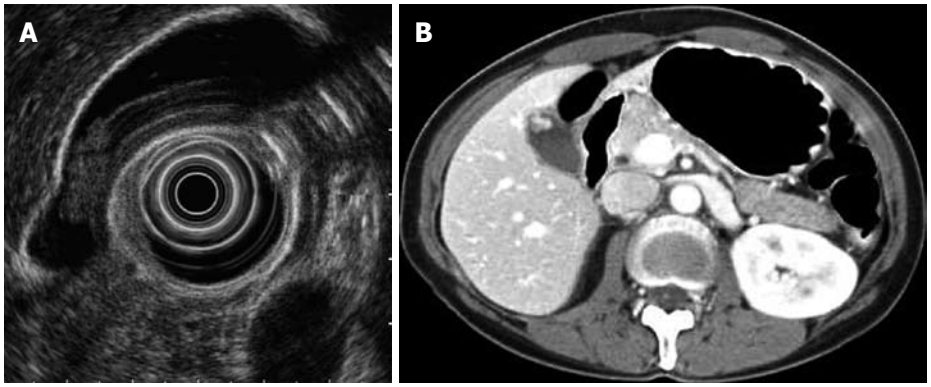

Figure 2 Misjudged case diagnosed as adenoma or carcinoma before surgery. A: EUS shows a 17.5-mm-diameter, homogeneously isoechoic, pedunculated mass; B: Abdominal CT shows an enhanced polypoid mass of the gallbladder in arterial phase. Histological examination of the surgical specimen showed a cholesterol polyp.

cases with polyps greater than 10 mm in diameter, EUS correctly distinguished 82.4% (14/17) of the cholesterol polyps, 87.5% (7/8) of the adenomyomatosis, and 88.9% (8/9) of the neoplastic lesions. The accuracy of EUS in diagnosing neoplastic lesions tended to be lower for polyps greater than 10 mm (79.7%) than for polyps less than 10 mm (83.3%) ($P = 0.12$). There was no significant difference between EUS and US in the diagnosis of cholesterol polyps.

Table 4 summarizes the results of differential diagnoses between neoplastic and benign polyps assessed by EUS and US. The overall sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy for EUS (US) in the diagnosis of neoplastic lesions were 66.7% (47.5%), 84.2% (72.0%), 50.0% (30.0%), 91.4% (84.4%), and 80.9% (67.0%), respectively (Table 5). When the results for relatively smaller polyps (diameter, < 10 mm) and for larger polyps (diameter, > 10 mm) were considered separately, the sensitivity, specificity, PPV, NPV, and accuracy for US were all lower than the values for EUS in both groups. The values for EUS in polyps less than 1.0 cm in diameter were lower than those in polyps greater than 1.0 cm in diameter.

DISCUSSION

Owing to the widespread use of conventional US, an increasing number of polypoid lesions of the GB are being identified. However, it is difficult to make differential diagnoses of polypoid GB lesions by US, CT, and magnetic resonance imaging. In general, factors that increase the probability that a GB polyp will be malignant include age greater than 50 years, a solitary lesion, a polyp greater than 1.0 cm in size, the presence of gallstones, a sessile lesion, and a rapid change in lesion size on serial ultrasonography[8,9]. All of these factors should be taken into consideration when advising patients with a polypoid lesion of the GB (PLG). The correct surgical management of PLGs is controversial. Although it is widely agreed that patients with symptomatic PLGs should be offered cholecystectomy, preferably by the laparoscopic route, the best treatment for an asymptomatic patient is not clear. In cases with a high probability of a malignant lesion, such as a PLG larger than 2 cm, open surgery is preferred to reduce the risk of tumor seeding associated with laparoscopic surgery. For asymptomatic PLGs smaller than 1 cm, follow-up US every 6 to 12 mo is necessary to exclude a rapidly growing malignant tumor[3].

There are a number of reports suggesting that sessile lesions smaller than 1.0 cm have an increased incidence of malignancy compared with those with a stalk[10]. In this study, 10 of 19 (52.6%) neoplastic polyps were pedunculated lesions smaller than 1 cm. Sugiyama *et al*[11] reported that approximately 30% of polyps with a diameter of 11-15 mm were cholesterol polyps and that about 40% of neoplastic polyps were 6-10 mm in diameter. Kubota *et al*[12] found that 57% of cholesterol polyps, 75% of adenomas, and 13% of neoplastic polyps were less than 10 mm in diameter. Thus, for polyps less than 10 mm in diameter, criteria other than size, along with an aggressive work-up, are needed to discriminate between neoplastic and non-neoplastic polyps.

EUS is considered to be superior to conventional US for imaging GB lesions, because EUS can provide high-resolution images of small lesions at higher ultrasound frequencies (7.5-12 MHz *vs* 3.5-5 MHz). Many

studies have investigated the relationship between the neoplastic nature of GB polyps and their morphological characteristics such as the number of polyps, the polyp shape, the diameter of the largest polyp, the echo level and internal echo pattern, and the polyp margin[12-14]. Among these variables, size is the most significant predictor of neoplastic polyps. However, the accuracy of EUS in identifying neoplastic lesions among polyps smaller than 10 mm in our study was only 44.4%, which was significantly lower than the identification rate among polyps greater than 1.0 cm (88.9%, $P < 0.05$).

To overcome the limitation of EUS in the differential diagnosis of neoplastic and non-neoplastic polypoid lesions less than 10-15 mm or less than 20 mm in size, an EUS scoring system has been adopted[7,15]. According to this system, the sensitivity, specificity, and accuracy of the risk for a neoplastic polyp were 81%, 86%, and 83.7%, respectively, for polyps with an EUS score of 6 or greater, whereas the sensitivity, specificity, and accuracy using a 10 mm cut-off diameter were 60%, 64%, and 62.7%, respectively[7]. Based on the EUS scoring system of Sadamoto *et al*[15], the sensitivity, specificity, and accuracy of the risk for neoplasia in polyps with scores of 12 or higher were 77.8%, 82.7%, and 82.9%, respectively. The EUS scoring system will be useful for differentiating between neoplastic and non-neoplastic polyps of the GB, however, the EUS variables used to calculate the score differ between the different EUS scoring systems.

The accuracy of EUS results tend to be lower for polyps smaller than 1 cm than for polyps greater than 1 cm in size. In our study, seven of 11 (63.6%) polyps less than 1.0 cm in size that were determined to be neoplastic by EUS before surgery were confirmed after surgery to be non-neoplastic lesions, including six cholesterol polyps and one adenomyomatosis. Using US, only two of 10 cases were determined to be neoplastic polyps after surgery. Thus, despite its higher accuracy compared with conventional US, EUS could not differentiate malignant from benign polyps smaller than 1.0 cm. No carcinoma was found in polyps less than 1.0 cm in size, but the prevalence of adenoma was 17.2% in our study.

Although EUS was more accurate than US, its accuracy for differentiating malignant from benign PLGs of less than 1.0 cm was low. EUS could not differentiate malignant lesions from benign polyps less than 1.0 cm in size, because such small polyps do not often show findings typical of cholesterol polyps, localized types of adenomyomatosis, or neoplastic lesions. Thus, EUS alone is not sufficient for determining a treatment strategy for PLGs of less than 1.0 cm. Polyps less than 1.0 cm in diameter without typical EUS or US findings should be followed-up by US at intervals of 6-12 mo. Changes in the size or structure of polypoid lesions should prompt reinvestigation with EUS and lead physicians to consider cholecystectomy.

COMMENTS

Background

The distinction between non-neoplastic, neoplastic and potentially malignant lesions is a major diagnostic dilemma and the therapeutic options for small polypoid lesions of the gallbladder remain controversial. Although endoscopic ultrasonography (EUS) was more accurate than ultrasonography (US), its accuracy for differentiating malignant from benign polypoid gallbladder lesions (PLGs) of less than 1.0 cm was low.

Research frontiers

Among many variables, size is the most significant predictor of neoplastic polyps. Although EUS was more accurate than US, its accuracy for differentiating malignant from benign PLGs of less than 1.0 cm was low. Thus, EUS alone is not sufficient for determining a treatment strategy for PLGs of less than 1.0 cm.

Innovations and breakthroughs

Many studies have investigated the relationship between the neoplastic nature of gallbladder (GB) polyps and their morphological characteristics such as the number of polyps, the polyp shape, the diameter of the largest polyp, the echo level and internal echo pattern, and the polyp margin. Among these variables, size is the most significant predictor of neoplastic polyps. However, the accuracy of EUS results tends to be lower for polyps smaller than 1 cm than for polyps greater than 1 cm in size. To overcome the limitation of EUS in the differential diagnosis of neoplastic from benign PLGs, an EUS scoring system has been adopted. The EUS scoring system will be useful for differentiating between neoplastic and non-neoplastic polyps of the GB, however, the EUS variables used to calculate the score differ between the different EUS scoring systems. Thus, EUS alone is not sufficient for determining a treatment strategy for PLGs of less than 1.0 cm.

Applications

The study results suggest that EUS alone is not sufficient for determining a treatment strategy for PLGs of less than 1.0 cm. Thus, new diagnostic criteria of EUS or tools are needed to distinguish between non-neoplastic, neoplastic, and potentially malignant lesions in small PLGs.

Terminology

Neoplastic gallbladder polyps: A neoplastic polyp has the properties of a neoplasm including adenoma and carcinoma. A non-neoplastic polyp does not have the properties of a neoplasm and includes cholesterol, inflammatory and fibrous polyps.

Peer review

Although retrospective, this study involves a large series of patients and describes the role of EUS well. The results suggest that EUS alone is not sufficient for determining a treatment strategy for PLGs of less than 1.0 cm and a new diagnostic approach is needed.

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BRIEF ARTICLES

Mucin gene expression in bile of patients with and without gallstone disease, collected by endoscopic retrograde cholangiography

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Abstract

AIM: To investigate the pattern of mucin expression and concentration in bile obtained during endoscopic retrograde cholangiography (ERC) in relation to gallstone disease.

METHODS: Bile samples obtained at ERC from 29 consecutive patients, 17 with and 12 without gallstone disease were evaluated for mucin content by gel filtration on a Sepharose CL-4B column. Dot blot analysis for bile mucin apoproteins was performed with antibodies to Mucin 1 (MUC1), MUC2, MUC3, MUC5AC, MUC5B and MUC6. Staining intensity score (0-3) was used as a measure of antigen expression.

RESULTS: MUC1, MUC2, MUC3, MUC5AC, MUC5B and MUC6 were demonstrated in 34.4%, 34.4%, 51.7%, 51.7%, 55.1% and 27.5% of bile samples, respectively. The staining intensity scores were 0.62 ± 0.94, 0.58 ± 0.90, 0.79 ± 0.97, 1.06 ± 1.22, 1.20 ± 1.26 and 0.41 ± 0.73, respectively. Mean mucin concentration measured in bile by the Sepharose CL-4B method was 22.8 ± 24.0 mg/mL (range 3.4-89.0 mg/mL). Mean protein concentration was 8.1 ± 4.8 mg/mL (range 1.7-23.2 mg/mL).

CONCLUSION: High levels of MUC3, MUC5AC and MUC5B are expressed in bile aspirated during ERC examination. A specific pattern of mucin gene expression or change in mucin concentration was not found in gallstone disease.

INTRODUCTION

Mucins are high-molecular-weight glycoproteins containing oligosaccharide side-chains attached to serine or threonine residues of the apomucin backbone by O-glycosidic linkages[1-4]. Several mucin (MUC) genes located on different chromosomes have been sequenced and cloned[5-14]. These genes encode apoproteins with specific tandem repeats of amino acids. Antibodies have been developed against the tandem repeats, enabling the identification of specific mucins by immunohistochemistry.

Mucins can be divided into two classes: gel-forming and membrane-associated. Bile mucin has two main domains: one rich in serine, threonine and proline, which contains the majority of the covalently-bound carbohydrates; and another, nonglycosylated domain, enriched in serine, glutamic acid, glutamine and glycine, which binds hydrophobic ligands such as bilirubin. An increased expression of gel-forming mucin, such as MUC5AC and MUC2, was found in patients with hepatolithiasis[15]. Although bile-duct mucin production has been extensively studied in malignant diseases[16-22], little is known about mucin synthesis and expression in cholelithiasis, choledocholithiasis and cholangitis.

The aim of the present study was to examine mucin concentration and specific expression in bile samples of patients undergoing endoscopic retrograde cholangiography (ERC) for the evaluation

of symptomatic bile duct disease, and to investigate the possible association between mucin expression and the clinical states of gallstone disease.

MATERIALS AND METHODS

Sampling

Twenty-nine patients who underwent ERC due to symptomatic bile duct disease were included in the study. Background data and results for ultrasound examinations and liver function tests were obtained from the files. Bile was collected by aspiration, as completely as possible, after the papilla was cannulated and before proceeding to any other procedures, such as papillotomy or choledochal stone removal. The Institutional Review Board (Ethical Committee) of Rabin Medical Center approved the study.

Bile analysis

To determine mucin concentration in the bile, we used the gel-filtration technique, as previously described[23,24]. Briefly, after centrifugation to remove debris, samples of bile were subjected to gel filtration on Sepharose CL-4B columns (1 × 40 cm). We used the closed-column system of Pharmacia Biotech (Cambridge, MA, USA): peristaltic pump, P-1; columns and adapters, C10; fraction collector, Redifrac and Ultraspec 1000; VV/visible spectrophotometer; and chart reader, 80-2109-03. Samples of 2 mL were applied to the columns and eluted with 10 mmol/L Tris-HCl buffer at pH 8.0. Fractions of 1 mL were collected, and optical density was determined at a wavelength of 280 nm. Findings were correlated with a standard curve of readings of mucin purified from porcine stomach (1% bound sialic acid), purchased from Sigma (St. Louis, MO, USA). The amount of protein was estimated by the Laury method.

Dot blot analysis

Samples were subjected to dot blot analysis on nitrocellulose membranes. Membranes were incubated with monoclonal antibodies to Mucin 1 (MUC1), MUC2, MUC3, MUC5AC, MUC5B and MUC6 (all mouse), followed by incubation with anti-mouse and IgG labeled with biotin. Antibody binding was detected with streptavidin-horseradish peroxide and chemiluminescent reagents (EZ-ECL, Beit-Haemek, Israel). Monoclonal antibodies were purchased from Neomarkers (Fremont, CA, USA). Staining intensity was scored (0-3) as a measure of antigen expression.

Statistical analysis

All results are expressed as mean ± SD. The analyses included descriptive statistics, χ^2 test, Student's *t*-test, and linear regression analysis. $P < 0.05$ was considered significant.

RESULTS

Study population

The study group consisted of 13 men and 16 women aged 64.5 ± 16.8 years (Table 1). A gallstone disease was diagnosed in 17 patients and excluded in 12 patients. The indications for ERC, abdominal ultrasound results, and liver function test results before ERC are presented in Table 1.

Table 1 Demographic and clinical data (n = 29)

Clinical parameter	n (%) or mean ± SD
Age	
mean ± SD (yr)	64.5 ± 16.8
Range	24-86
Sex	
Men	13 (44.8)
Women	16 (55.2)
Main indication for ERC	
Cholestasis/jaundice	16 (55.2)
Choledocholithiasis/dilated CBD	5 (17.2)
Cholangitis	3 (10.3)
SOL of papilla	3 (10.3)
Unresolved pancreatitis	2 (6.9)
Abdominal ultrasound results	
Dilated CBD	15 (51.7)
Cholelithiasis	12 (41.4)
Dilated intrahepatic ducts	10 (34.5)
Choledocholithiasis	3 (10.3)
Pancreatitis	1 (3.4)
CBD width (mm)	
mean ± SD	8.7 ± 3.7
Range	6-18
Liver function tests, mean ± SD (range)	
Total bilirubin (mg/dL)	4.5 ± 6.9 (0.3-32)
Direct bilirubin (mg/dL)	3.0 ± 4.9 (0.1-23)
Alanine aminotransferase (U/L)	206.5 ± 243.3 (12-895)
Aspartate aminotransferase (U/L)	156.4 ± 185.6 (15-812)
Gamma glutamyl transpeptidase (U/L)	369.4 ± 370.4 (13-1337)
Alkaline phosphatase (U/L)	291.0 ± 371.4 (56-1893)

ERC: Endoscopic retrograde cholangiography; CBD: Common bile duct; SOL: Space-occupying lesion.

ERC results

The ERC findings are shown in Table 2. Linear regression analysis revealed a positive correlation between the mean common bile duct (CBD) width measured on abdominal ultrasound and ERC. There was also a positive correlation between ultrasound findings of cholelithiasis and ERC findings of dilated CBD; between the presence of a clinical syndrome of cholangitis and ultrasound findings of pancreatitis; and between increased concentrations of serum direct bilirubin and ERC findings of CBD stricture. A wider CBD was demonstrated in patients with evidence of choledocholithiasis on ERC (10.90 ± 4.97 mm) than in patients without CBD stones (7.44 ± 1.98 mm). Information from the ultrasound studies and ERC results was used to stratify the patients into a group with gallstone related disease, and a group without gallstone disease.

Mucin concentration in bile

Mean ± SD mucin concentration in bile, measured by the Sepharose CL-4B method, was 22.8 ± 24.0 mg/mL (range 3.4-89.0 mg/mL). Mean protein concentration was 8.1 ± 4.8 mg/mL (range 1.7-23.2 mg/mL). Mucin concentration in bile was not significantly different between men and women (24.68 ± 27.29 mg/mL *vs* 21.38 ± 21.96 mg/mL), patients younger or older than 70

Table 2 Results of ERC (n = 29)

Clinical parameter	n (%) or mean ± SD
Diagnosis	
CBD width (mm)	9.4 ± 4.0
Range	6-18
Choledocholithiasis	11 (37.9)
Pigmented stones	4 (13.8)
Cholelithiasis	5 (17.2)
Enlarged papilla	7 (24.1)
Dilated CBD	15 (51.7)
CBD stricture	4 (13.8)
Intrahepatic ducts dilation & stricture	3 (10.3)
Torn papilla	3 (10.3)
Bile leakage	1 (3.4)
Mirizzi syndrome	1 (3.4)
Treatment	
Sphincterotomy	16 (55.2)
Biopsy of the papilla	5 (17.2)
Stent insertion	2 (6.9)
Cholecystostomy	1 (3.4)

years (18.87 ± 15.72 mg/mL *vs* 26.59 ± 30.07 mg/mL), and patients with or without choledocholithiasis (22.46 ± 24.94 mg/mL *vs* 23.11 ± 24.29 mg/mL).

Mucin expression in bile

The expression of the mucin genes examined by dot blot analysis is shown in Table 3. Linear regression analysis revealed a positive correlation between MUC5AC and MUC5B expression [MUC5B = 0.273 + (0.874 × MUC5AC); R = 0.845]. There was also a positive correlation between MUC1 expression and papillary enlargement on ERC. The correlation between the expressions of the different MUC genes in bile is shown in Table 4.

Comparison of patients with and without gallstone disease

Summarizing the clinical and imaging data allowed the patients to be stratified into a group with diagnosed gallstone disease (n = 17), and a group with no evidence of gallstone disease (n = 12). There were no significant differences in gender, age, laboratory results, ultrasound finding, indication and results of ERC, except in the presence of gallstone disease (Table 5). Mucin concentration in bile was similar in both groups (21.68 ± 7.87 mg/mL *vs* 24.54 ± 24.10 mg/mL, P = 0.759), as was mucin gene expression (Table 5).

Table 3 Mucin gene expression in bile collected in ERC

Mucin gene	Score mean ± SD (range)	Cases (%)
MUC1	0.62 ± 0.94 (0-3)	34.4
MUC2	0.58 ± 0.90 (0-3)	34.4
MUC3	0.79 ± 0.97 (0-3)	51.7
MUC5AC	1.06 ± 1.22 (0-3)	51.7
MUC5B	1.20 ± 1.26 (0-3)	55.1
MUC6	0.41 ± 0.73 (0-2)	27.5

Table 4 Correlation between the expression of the different mucin genes in bile collected by ERC

Mucin gene	Correlation with mucin gene	P value
MUC1	MUC2	0.0001
	MUC3	0.0001
	MUC5AC	0.0125
	MUC5B	0.049
	MUC6	0.0001
MUC2	MUC3	0.0001
	MUC5AC	0.0080
	MUC5B	0.0001
	MUC6	0.0001
MUC3	MUC5AC	0.0031
	MUC6	0.0003

DISCUSSION

Different mucin genes are expressed in bile, and the role of each is unclear. Bile mucin is derived from pure hepatic bile, gallbladder-concentrated bile, and mucin secreted by the bile duct epithelium. Ko *et al*[25] found that in patients with biliary sludge, mucin concentration was higher in bile collected by ERC than in gallbladder bile. They concluded that the biochemical composition of hepatic bile is modified during residence in the gallbladder, contributing to sludge formation, and that hepatic bile samples are therefore inappropriate for microscopic detection of microlithiasis. However, although the mucin concentration in hepatic bile in the present study was similar to that reported by Ko *et al*[25] (22.8 ± 24.0 mg/mL *vs* 20 ± 30 mg/mL), the concentration of mucin in gallbladder bile in our previous study was 17.5 ± 16.4 mg/mL[26], close to that of hepatic bile and much lower than the 450 ± 290 mg/mL found by Ko *et al*[25]. Thus, our studies do not support the assumption of Ko *et al*[25], and this controversy requires further investigation.

We demonstrated a higher expression of two secretory mucin proteins, MUC5AC and MUC5B, and the membrane-bound protein, MUC3. MUC5AC and MUC5B are both gel-forming mucins that may increase the viscosity of bile in cases of symptomatic bile duct disease. Since we could not find a change in mucin concentration or in these specific genes expressions in bile derived from patients with or without gallstone disease, our findings do not support a role for MUC5AC or MUC5B in the etiopathogenesis of gallstones.

Zen and coworkers described a lipopolysaccharide-induced increase in MUC2 and MUC5AC expression in cultured murine biliary epithelial cells, which was mediated by tumor necrosis factor alpha[27]. They concluded that since lipopolysaccharide is a bacterial component, bacterial infection may be involved in the altered mucin secretion in the intrahepatic biliary tree and, thereby, in the lithogenesis of hepatolithiasis. Wandenhaute and coworkers noted a strong mRNA expression of MUC5B, MUC3 and MUC6, and a weak expression of MUC1, MUC2 and MUC5AC, in biliary epithelial cells[28]. Lee and Liu found that MUC3 and MUC5B were the main mucin genes expressed in the biliary epithelium of stone-containing intrahepatic bile ducts and normal controls[29]. Mucin gene expression

Table 5 Comparison between patients with (n = 17) and without (n = 12) gallstones

	Gallstones diseas n (%)	No evidence for gallstones n (%)	P value
Age, mean ± SD (years)	61.35 ± 20.13	69.00 ± 9.58	0.234
Sex (men)	8 (47.1)	5 (41.7)	0.927
Main indication for ERC			
Jaundice	7 (41.1)	9 (75)	0.153
Dilated CBD	4 (23.5)	1 (8.0)	0.554
Cholangitis	3 (20.0)	0	0.288
SOL of papilla	1 (6.0)	2 (17.0)	0.737
Unresolved pancreatitis	2 (12.0)	0	0.288
Abdominal ultrasound results			
Dilated CBD	10 (59.0)	5 (42)	0.599
Cholelithiasis	12 (70.6)	0	< 0.0001
Dilated intrahepatic ducts	6 (40)	4 (30)	0.873
Choledocholithiasis	3 (17.6)	0	0.360
Pancreatitis	1 (5.9)	0	0.861
CBD width (mm), mean ± SD	9.65 ± 4.39	7.50 ± 2.24	0.132
Liver function tests, mean ± SD			
Total bilirubin (mg/dL)	5.12 ± 7.83	3.68 ± 5.52	0.589
Direct bilirubin (mg/dL)	3.38 ± 5.53	2.55 ± 4.03	0.662
Alanine aminotransferase (U/L)	245.41 ± 270.76	151.58 ± 196.14	0.315
Aspartate aminotransferase (U/L)	167.29 ± 150.54	141.08 ± 233.11	0.715
Gamma glutamyl transpeptidase (U/L)	443.47 ± 388.13	264.50 ± 331.14	0.206
Alkaline phosphatase (U/L)	352.88 ± 449.95	185.08 ± 174.44	0.323
ERC diagnosis			
CBD width (mm), mean ± SD	9.59 ± 3.99	9.17 ± 4.37	0.790
Choledocholithiasis	11 (64.7)	0	0.002
Pigmented stones	4 (24.0)	0	0.198
Cholelithiasis	5 (29.0)	0	0.122
Enlarged papilla	3 (16.0)	4 (33.0)	0.533
Dilated CBD	10 (60.0)	5 (40.0)	0.494
CBD stricture	1 (10.0)	3 (30.0)	0.376
Torn papilla	3 (17.6)	0	0.360
Treatment			
Sphincterotomy	12 (71.0)	4 (33.0)	0.099
Biopsy of the papilla	1 (10.0)	4 (33.0)	0.288
Mucin gene score, mean ± SD			
Mucin concentration (mg/mL)	21.68 ± 7.87	24.54 ± 24.1	0.759
Protein concentration (mg/mL)	7.87 ± 4.53	8.61 ± 5.48	0.694
MUC1	0.59 ± 0.87	0.66 ± 1.07	0.848
MUC2	0.53 ± 0.80	0.66 ± 1.07	0.711
MUC3	0.88 ± 0.93	0.66 ± 1.07	0.560
MUC5AC	0.47 ± 0.80	0.33 ± 0.65	0.621
MUC5B	1.23 ± 1.25	0.83 ± 1.19	0.394
MUC6	1.29 ± 1.26	1.08 ± 1.31	0.667

was altered in dysplastic preneoplastic cells.

The main weakness of our study is the absence of healthy controls. We could not compare mucin concentration and gene expression in the cholestatic situation with that of normal bile collected in ERC, since ERC is usually performed with therapeutic intent in symptomatic patients.

In the present study, we observed a positive correlation between MUC1 expression in bile and the expression of all the other mucin genes examined. Wang and coworkers reported a similar result in mice[30]. They described a positive correlation between MUC1 and MUC5AC expression, indicating a gene-gene interaction that might affect the accumulation of mucin gel and cholesterol gallstone formation.

In summary, we could not demonstrate a change in mucin secretion and expression between patients with and without gallstone disease, or support the role of mucin in the etiopathogenesis of biliary sludge or stone formation.

COMMENTS

Background

Secretory mucins are gel-forming and may increase bile viscosity. The biochemical composition of hepatic bile is modified during residence in the gallbladder, contributing to sludge formation. An increased expression of gel-forming mucin, such as MUC5AC and MUC2, was found in patients with hepatolithiasis. Little is known about mucin synthesis and expression in cholelithiasis, choledocholithiasis and cholangitis.

Innovations and breakthroughs

High levels of MUC3, MUC5AC and MUC5B are expressed in bile aspirated during endoscopic retrograde cholangiography examination. A specific pattern of mucin gene expression or change in mucin concentration was not found in gallstone disease.

Applications

Expression of other mucin genes or changes in concentration should be investigated in gallstone disease. The role of mucin synthesis and secretion in gallstone formation is still unknown.

Peer review

The manuscript by Vilkin *et al* describes the analysis of certain members of the Mucin gene family in the bile of patients with and without gallstone disease. The authors demonstrate the presence of Mucin 1 (MUC1), MUC2, MUC3, MUC5AC, MUC5B and MUC6 in the bile of all patients, but there was no correlation to

the presence of gall stones. While this manuscript contains negative data, with no conclusive outcomes, the data is nevertheless important as it disproves a currently regarded theory about the role of mucins in gall stone formation.

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BRIEF ARTICLES

Determination of correlation of Adjusted Blood Requirement Index with outcome in patients presenting with acute variceal bleeding

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Abstract

AIM: To determine the correlation of Adjusted Blood Requirement Index (ABRI) with the 7th day outcome in patients presenting with acute variceal bleeding.

METHODS: All patients presenting with acute variceal hemorrhage (AVH) were included. Patients with previous band ligation, sclerotherapy, gastrointestinal or hepatic malignancies were excluded. Patients were managed as per standard protocol for AVH with terlipressin and band ligation. ABRI scores were calculated using the formula outcome of alive or expired up to the 7th day after treatment. The correlation between ABRI and mortality was estimated and a receiver operative characteristic (ROC) curve was plotted.

RESULTS: A total of 113 patients (76 male; 37 female) were included. On assessment, 18 were in Child's Pugh Class A, 82 in Class B and 13 were in Class C. The median number of blood units transfused ± inter-quartile range was 3.0 ± 2.0. The median ± inter-quartile range for ABRI was 1.3 ± 1.1. The ROC curve of ABRI for expiry showed a significantly large area of 0.848 ($P < 0.0001$; 95% CI: 0.75-0.95). A significant correlation of log transformation of ABRI with an outcome of mortality was present ($P < 0.0001$).

CONCLUSION: ABRI correlates strongly with mortality.

INTRODUCTION

Chronic liver diseases and cirrhosis are now being recognized as an important cause of morbidity and mortality worldwide. Acute variceal hemorrhage (AVH) secondary to cirrhosis is to date the most important cause of mortality in cirrhosis[1]. In Pakistan, hepatitis B and C are the most important causes of cirrhosis[2]. The frequency of varices is very high in cirrhotic patients, nearly 40% of patients with compensated cirrhosis and 60% with decompensated cirrhosis have varices[3]. Due to recent advancements, mortality from AVH has been reduced to 20% from the first variceal bleed[4]. Bleeding from AVH carries a high risk of mortality during the first 5 d, with a gradual decline in risk over the next 4-6 wk[5]. The prediction and evaluation of adequate hemostasis by non-endoscopic methods are desired by treating physicians. Many criteria and definitions to evaluate failure to control and prevent variceal bleeding were developed in the Baveno Consensus Workshops Ⅰ-Ⅲ but failed in clinical application due to cumbersome procedures and calculations[6-10]. Further developments in this subject identified an independent factor the "Adjusted Blood Requirement Index (ABRI)" in the Baveno Workshop Ⅳ[11]. ABRI was developed to determine adequate control or failure to control variceal hemorrhage. An ABRI value of $\geqslant 0.75$ at any point time was defined as a failure to control variceal bleeding[11].

Failure of AVB control leads to increased mortality. Thus ABRI could be used to assess the risk of mortality. A correlation between ABRI and mortality has not been evaluated in a prospectively designed study. We have reported its correlation with outcome in a retrospective analysis previously[12]. As there are no reports of a prospective evaluation of ABRI and its relation to mortality, there is a need to assess this correlation in our settings.

This study was designed to evaluate the correlation between ABRI and outcome at the 7th day after hospital admission as improved or expired in acute variceal bleeding.

MATERIALS AND METHODS

All cirrhotic patients who presented with AVB were included. Informed consent was obtained from all patients. Patients with a history of previous band ligation or sclerotherapy, hepatocellular carcinoma and the presence of peptic ulcer or gastrointestinal (GI) malignancy on endoscopy were excluded. Patients were managed as per standard protocol of acute variceal bleeding[10]. Blood samples were taken for Complete Blood Counts, Prothrombin Time, Liver Function Tests and albumin before the start of therapy. Child's Pugh Class assessment was carried out. All patients were given terlipressin 2 mg *iv* initial dose and followed by 1 mg/6 h for 3 d. The number of blood units transfused was noted and endoscopic variceal band ligation (EVBL) was performed within 24 h of admission. Study endpoint was patient outcome (alive or expired at the 7th day after admission). The ABRI value was calculated using the following formula[11]: ABRI = blood units transfused/[(final hematocrit - initial hematocrit) + 0.01]. Child's Pugh score was calculated using the formula[13] shown in Table 1.

Sample size

Sample size was estimated using the following parameters: Level of Significance (α) = 5%; Power of test (1-β) = 80%; Test value of population proportion (P_0) = 20% (0.2); Anticipated value of population proportion (P_a) = 30% (0.3); Sample size (n) = 109.

Statistical analysis

mean ± SD was calculated for age. Median and inter-quartile range were calculated for the number of blood units transfused and ABRI. Frequencies of gender, Child's Pugh Class and outcome were calculated. ABRI values $\geqslant$ 0.75 were recoded into a new variable as uncontrolled while ABRI values < 0.75 were recoded as controlled and their frequency estimated. χ^2 test was performed for outcome with ABRI control status and Child's Pugh Class was carried out with continuity correction and likelihood ratio applied where indicated. A receiver operative characteristic (ROC) curve of ABRI was plotted for expiry. Log transformation of variable ABRI was carried out as it was not normally distributed and then used for Pearson's Bivariate correlation with outcome. The significance level was set at $P \leqslant 0.05$. SPSS version 17.0 was used for statistical analysis.

Table 1 Child's Pugh score was calculated (using formula)

Parameter	Numerical score		
	1	2	3
Ascites	None	Slight	Moderate to severe
Encephalopathy	None	Slight to moderate	Moderate to severe
Bilirubin (mg/dL)	< 2.0	2-3	> 3.0
Albumin (g/dL)	> 3.5	2.8-3.5	< 2.8
Prothrombin time (prolonged in seconds)	1-3 s	4-6 s	> 6.0

Child's Pugh Class A = 5-6 points; Child's Pugh Class B = 7-9 points; Child's Pugh Class C = 10-15 points.

Table 2 Cross tabulation of ABRI groups with outcome

		Outcome		Total
		Alive	Expired	
ABRI groups	Controlled	27	0	27
	Uncontrolled	67	19	86
Total		94	19	113

RESULTS

A total of 113 patients fulfilling the inclusion/exclusion criteria were inducted. These included 76 (67.3%) male (44.3 ± 11.8 years) and 37 (32.7%) female (44.1 ± 9.4 years). Terlipressin was given to 111 patients (98.2%) immediately on admission. EVBL was performed in 105 (92.9%) patients. The assessment on admission showed that 18 (15.9%) were in Child's Pugh Class A; 82 (72.6%) in Child's Pugh Class B and 13 (11.5%) were in Child's Pugh Class C. The median number of blood transfusions given was 3.0 pints and the inter-quartile range was 2.0. The median ABRI was 1.3 with an inter-quartile range of 1.1. The number of patients with ABRI $\geqslant$ 0.75 was 86 (76.1%) showing a failure to control variceal bleeding according to ABRI criteria. Outcome at the 7th day after admission showed that 94 (83.2%) patients were alive while 19 (16.8%) patients had expired during this period. Cross tabulation of outcome (alive and expired) with ABRI status [controlled (< 0.75) and uncontrolled ($\geqslant$ 0.75)] showed that no patients had expired in the ABRI controlled group (Table 2). χ^2 test with continuity correction gave a significance value of $P = 0.017$. A similar cross tabulation with Child's Pugh Class showed that the highest percentage of patients expired in Child's Pugh Class C while no patients with Child's Class A expired (Table 3). χ^2 test with the Likelihood Ratio gave significant differences in the frequencies of expiry with Child's Pugh Class ($P < 0.0001$). A ROC curve was plotted using expiry as a state variable (Figure 1). The area under the curve was significantly large at 0.848 ($P < 0.0001$; 95% CI: 0.75-0.95). The sensitivity and specificity of the ABRI cutoff value of 0.75 in our study was 100% and 73.4%, respectively. The correlation of

Table 3 Cross tabulation of Child's Pugh Class with outcome

			Outcome		Total
			Alive	Expired	
Child's Pugh Class	Class A	Count	18	0	18
		% within Child's Pugh Class	100.0%	0.0%	100.0%
	Class B	Count	71	11	82
		% within Child's Pugh Class	86.6%	13.4%	100.0%
	Class C	Count	5	8	13
		% within Child's Pugh Class	38.5%	61.5%	100.0%
Total		Count	94	19	113
		% within Child's Pugh Class	83.2%	16.8%	100.0%

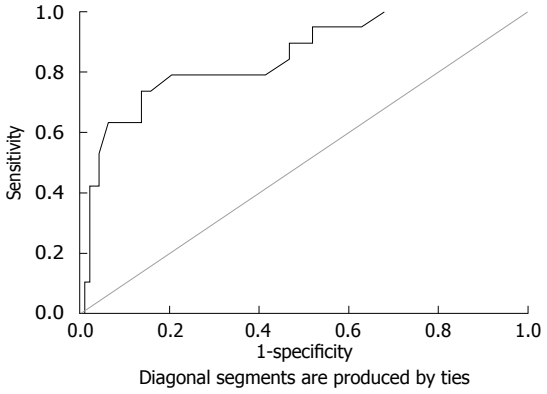

Figure 1 ROC curve of ABRI against expiry.

ABRI with outcome was analyzed by Pearson's Bivariate Correlation test. As the data of ABRI was skewed and not normally distributed its Log_{10} transformation was used. The results showed a significant correlation between ABRI and expiry with $P < 0.0001$.

DISCUSSION

This study showed a significant correlation between ABRI and the 7th day outcome in patients with AVH. This is a very significant finding as it is important to predict the outcome at an initial stage of management and ABRI suggests whether the variceal hemorrhage has been arrested at any point during management. Our study also established its correlation with mortality. Earlier validation studies of ABRI were retrospective[11,12]. The current study is prospective and designed more specifically to assess the correlation of ABRI with mortality which has not been previously studied. The correlation of higher ABRI scores with mortality was significant and this simple to use parameter should be used to assess failure to control bleeding and risk of mortality. The number of units of blood transfused and hematocrit levels, if used alone, are not good criteria to assess variceal bleeding control. We also used pharmacological and endoscopic interventions and the combined effect of these interventions was reflected in the outcome which was also observed in other reports from this region[14]. About 70% of patients rebleeding within 2 years, thus managing the index bleed properly and obliteration of varices can decrease rebleeding[15,16].

Many scoring systems have been derived to predict the outcome of upper GI hemorrhage. The Rockall score is one such scoring system for predicting rebleeding and mortality which also showed good correlation[17,18]. Limitations of the Rockall score are that it is rather difficult to use with the requirement of more parameters as compared to ABRI and it is not variceal bleeding specific, but designed for both variceal and non-variceal bleeding[18,19]. Another popular scoring system, the Child's Pugh score predicts all cause morbidity and mortality in cirrhotic patients but is not specific for variceal hemorrhage[20].

In conclusion, among the many predictive scoring systems in cirrhotic patients, ABRI is specific for variceal hemorrhage and correlates strongly with mortality and is a good indicator of the failure of variceal hemorrhage control.

COMMENTS

Background

In a developing country like Pakistan, hepatitis B and C are the most important causes of cirrhosis. The frequency of varices is very high in cirrhotic patients, nearly 40% of patients with compensated cirrhosis and 60% of patients with decompensated cirrhosis have varices. Mortality from variceal bleeding is still high at 20%. The prediction of mortality risk is difficult and available scores are difficult to calculate and thus do not enjoy wide acceptability and application.

Research frontiers

Adjusted Blood Requirement Index (ABRI) is a score which is used to determine the failure to control variceal bleeding. In this study it was correlated with the outcome of mortality.

Innovations and breakthroughs

Many scoring systems are in use to predict the outcome of upper gastrointestinal hemorrhage. The Rockall score is one such scoring system for predicting rebleeding and mortality but it is rather difficult to use. Another scoring system, the Child's Pugh score predicts all cause morbidity and mortality in cirrhotic patients but is not specific for variceal hemorrhage. The ABRI is a variceal hemorrhage-specific score and is easy to use.

Applications

ABRI: A validated tool to determine variceal bleeding control also correlates well with mortality in such patients.

Peer review

Many scoring systems have been described to predict the prognosis of upper gastrointestinal hemorrhage like Rockall and Child's Pugh but these have limitations. In practice, it is useful to predict the outcome at admission of patients with acute variceal hemorrhage. This study showed a significant correlation between ABRI and expiry. The methodology is correct. This work deserves to be published to stimulate other teams over the world to perform the same study with a large number of patients.

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BRIEF ARTICLES

Local anesthesia with ropivacaine for patients undergoing laparoscopic cholecystectomy

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Abstract

AIM: To investigate the effect of pain relief after infusion of ropivacaine at port sites at the end of surgery.

METHODS: From October 2006 to September 2007, 72 patients undergoing laparoscopic cholecystectomy (LC) were randomized into two groups of 36 patients. One group received ropivacaine infusion at the port sites at the end of LC and the other received normal saline. A visual analog scale was used to assess postoperative pain when the patient awakened in the operating room, 6 and 24 h after surgery, and before discharge. The amount of analgesics use was also recorded. The demographics, laboratory data, hospital stay, and perioperative complications were compared between the two groups.

RESULTS: There was no difference between the two groups preoperatively in terms of demographic and laboratory data. After surgery, similar operation time, blood loss, and no postoperative morbidity and mortality were observed in the two groups. However, a significantly lower pain score was observed in the patients undergoing LC with local anesthesia infusion at 1 h after LC and at discharge. Regarding analgesic use, the amount of meperidine used 1 h after LC and the total used during admission were lower in patients undergoing LC with local anesthesia infusion. This group also had a shorter hospital stay.

CONCLUSION: Local anesthesia with ropivacaine at the port site in LC patients significantly decreased postoperative pain immediately. This explains the lower meperidine use and earlier discharge for these patients.

Key words: Prospective randomized trial; Local anesthesia; Ropivacaine; Normal saline; Laparoscopic cholecystectomy

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INTRODUCTION

Since 1987, laparoscopic cholecystectomy (LC) has been the favored treatment for gallbladder lesions[1]. Historically, contraindications to LC have included obesity, pregnancy, acute cholecystitis, and cardiovascular disease. With experience gained from laparoscopic surgery, LC has been attempted successfully and has become the procedure of choice in each subgroup of patients[2,3]. A major benefit of laparoscopy in upper gastrointestinal surgery results from avoidance of an upper abdominal incision. LC has proven benefits of less pain and improved pulmonary function tests compared with small-incision cholecystectomy[4-6]. However, assessment of postoperative stay and return to normal activity has shown conflicting results[5-7]. Many trials have assessed methods of reducing pain after LC, and several aspects of pain

after laparoscopy have been addressed[8-11]. These studies have concentrated on the mechanism of pain or focused on gynecological procedures, with an emphasis on the role of non-steroidal anti-inflammatory drugs (NSAIDs)[8]. Some of the reviews have demonstrated the heterogeneity of randomized controlled trials and have concluded that pain after LC is multifactorial[11]. Although, many methods of analgesia produce short-term benefits, this does not equate with earlier discharge or improved postoperative function.

This prospective and randomized controlled trial aimed to clarify the impact of infusion of local analgesia at the port site after LC on pain relief and postoperative outcome.

MATERIALS AND METHODS

From October 2006 to September 2007, 72 patients undergoing LC by the authors (Yeh CN, Yeh TS, and Chao TC) at the Department of Surgery, Chang Gung Memorial Hospital, Taiwan were included in this prospective randomized controlled trial. The study subjects were adult patients who had been referred for elective LC for gallbladder lesions. The diagnostic work-up for patients with gallbladder lesions before LC included history taking, physical examination, abdominal ultrasonography, abdominal computed tomography, and magnetic resonance cholangiopancreatography. The study was approved by the local institutional review board of Chang Gung Memorial Hospital and all patients gave informed consent before taking part in the study.

Randomization and treatment

The randomization was centralized and used a random permuted block design. Eligible patients were aged 20-85 years, not pregnant, and had adequate hematological, hepatic and renal function. The exclusion criteria were as follows: immunosuppressive drug therapy within the previous 6 mo; an immunosuppressive condition, including AIDS; autoimmune disorders; organ transplantation; radiotherapy or chemotherapy within the previous 6 mo; and insulin-dependent diabetes mellitus (type 1). Discharge from the wards was the primary endpoint. Clinical features, laboratory data, operative outcomes, pain score, and analgesic requirement were analyzed and compared between the ropivacaine and saline groups. Hospital stay was defined as the number of days from operation to the actual date of hospital discharge. Surgical mortality was defined as death that occurred within 1 mo after surgery.

Seventy-two patients were included in the study and randomized into a control or local anesthesia with ropivacaine (LA) group. All 72 patients received general anesthesia with the same protocol by one of the authors (Lin CC, anesthesiologist). The LA group received 1.0% ropivacaine 20 mL at the port site after wound closure (6 mL for epigastric port, 6 mL for umbilical port, and 4 mL for each working port). The control group received 0.9% normal saline 20 mL at the port site after wound closure (6 mL for epigastric port, 6 mL for umbilical port, and 4 mL for each working port). Ropivacaine or normal saline was applied to the skin, subcutis, fascia, and parietal peritoneum through the port sites at the end of surgery.

Table 1 Demographic data of 72 patients undergoing LC with and without local anesthesia infusion at the port site

	Control (n = 36)	LA (n = 36)	P
Age (yr)	48.4 ± 13.0	50.6 ± 12.4	0.461
Gender (M:F)	13:23	6:30	0.061
Previous abdominal operation history (+)	10 (27.8)	11 (30.6)	0.795
Associated disease (+)	16 (44.4)	12 (33.3)	0.334
Diagnosis			0.991
Gall stone	25 (69.4)	26 (72.2)	
Gall stone and AC	3 (8.3)	3 (8.3)	
Gall stone and CC	6 (16.7)	5 (13.9)	
Gall bladder polyp	2 (5.6)	2 (5.6)	
ASA grade	1.7 ± 0.6	1.5 ± 0.6	0.106
Operation time (min)	84.7 ± 31.3	78.5 ± 33.1	0.417
Blood loss (cc)	35.9 ± 84.8	32.4 ± 58.2	0.688
Conversion rate	0	0	NA
Post-operative drain	3 (8.3)	4 (11.1)	0.691
Morbidity rate	0	0	NA
Mortality rate	0	0	NA
Hospital stay (d)	2.8 ± 2.7	1.1 ± 0.3	0.001

LC: Laparoscopic cholecystectomy; M: Male; F: Female; AC: Acute cholecystitis; CC: Chronic cholecystitis; NA: Not available.

Patient monitoring and testing

A visual analog scale (VAS) with a 10-cm vertical score ranged from "no pain" to "worst possible pain". The VAS was used to assess postoperative pain when the patient awakened in the operating room (about 1 h after surgery), then after 6 and 24 h, and before discharge. The pain score was recorded by the authors (Lee HL, Liu YY, Wang SY, Tsai CY, and Yeh CN). Pain intensity was estimated using a VAS and the amount of analgesics used. The biochemistry data, operative time, hospital stay, and perioperative complications were recorded.

Statistical analysis

All data are presented as the percentage of patients or mean ± SD. Numerical data were compared by independent two-sample t test or paired two-sample t test. Pearson χ^2 test and Fisher exact test were used for nominal variables. All statistical analyses were performed using the SPSS computer software (Chicago, IL, USA). $P < 0.05$ was considered to be statistically significant.

RESULTS

Clinical features, laboratory data, and operative outcomes

Table 1 summarizes the demographic data of patients with gallbladder lesions receiving LC without local anesthesia (control group) and with local anesthesia (LA group). Both groups shared a similar age distribution and sex ratio. The two groups displayed no significant difference in ratio of previous abdominal operation, etiology of disease, and operative indications. The LA group had similar American Society of Anesthesiologists

Table 2 Laboratory data of 72 patients undergoing LC with and without local anesthesia infusion at the port site (mean ± SD)

	Control (n = 36)	LA (n = 36)	P
Hemoglobin (g/dL)	13.4 ± 1.8	14.1 ± 1.7	0.634
WBC (/μL)	7317.1 ± 2898.6	6617.3 ± 2226.8	0.681
BUN (mg/dL)	14.7 ± 8.5	14.1 ± 3.4	0.634
Creatinine (mg/dL)	1.1 ± 0.7	0.9 ± 0.1	0.130
Bilirubin (direct) (mg/dL)	0.27 ± 0.14	0.26 ± 0.14	0.713
Bilirubin (total) (mg/dL)	0.87 ± 0.77	0.66 ± 0.32	0.158
AST (IU/L)	29.8 ± 31.7	21.9 ± 18.8	0.206
ALT (IU/L)	36.9 ± 48.8	26.8 ± 25.1	0.269
ALP (IU/L)	86.2 ± 88.4	74.1 ± 38.8	0.422

BUN: Blood urea nitrogen; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase.

Table 3 Difference in pain scale and analgesia use of 72 patients undergoing LC (control *versus* local anesthesia) (mean ± SD)

	Control (n = 36)	LA (n = 36)	P
Pain analogue scale			
1-h post LC	6.8 ± 2.2	5.6 ± 2.0	0.021
6-h post LC	4.5 ± 1.7	3.9 ± 1.5	0.112
24-h post LC	2.7 ± 1.5	2.1 ± 1.1	0.039
Discharge post LC	1.7 ± 0.8	1.3 ± 0.6	0.020
Meperidine requirement (mg)			
1-h post LC	25.9 ± 21.3	13.0 ± 16.8	0.006
6-h post LC	22.9 ± 21.3	16.2 ± 26.5	0.347
24-h post LC	11.4 ± 30.0	5.4 ± 19.7	0.314
Discharge post LC	5.7 ± 26.5	0	0.211
Total amount	65.9 ± 79.7	34.6 ± 37.8	0.040
Acetaminophen requirement (500 mg/tablet)			
1-h post LC	0.06 ± 0.24	0.05 ± 0.23	0.955
6-h post LC	0.60 ± 0.74	0.32 ± 0.58	0.081
24-h post LC	0.57 ± 0.92	0.54 ± 0.80	0.879
Discharge post LC	0.20 ± 0.53	0.03 ± 0.16	0.073
Total amount	1.43 ± 1.56	0.95 ± 1.13	0.139

grade, operative time, operative blood loss, postoperative drain insertion, and complication rates as the control group. No 30-d mortality occurred in this study. The LA group had a significantly shorter hospital stay than the control group (1.1 ± 0.3 d *vs* 2.8 ± 2.7 d, $P = 0.001$). Table 2 displays the laboratory data of the 72 patients. No significant difference was noted between the two groups.

Evaluation of pain relief

Table 3 and Figure 1 compare the pain intensity and analgesic requirement between the control and LA groups. Both groups achieved gradual pain relief after surgery in terms of VAS for pain and need for analgesics. However, the LA group experienced significantly less pain at 1 and 24 h after surgery and at discharge when compared with the control group. Furthermore, the LA group had less meperidine use at 1 h and total meperidine use after LC. However, there was no significant difference in acetaminophen use between the two groups (Figures 2-4).

DISCUSSION

Postoperative pain associated with LC is less intense and lasts a shorter time than that seen with open cholecystectomy. This explains why patients can be discharged and returned to their normal daily activities earlier[12]. However, as seen in this study, LC is not a pain-free procedure. Pain remains a prevalent complaint of the

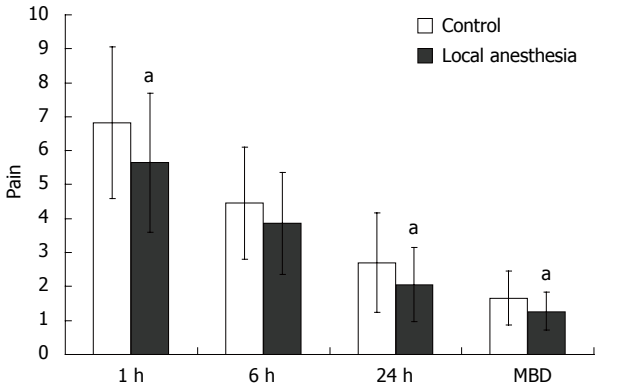

Figure 1 Pain evaluation of LC patients in the LA and control groups. $^aP < 0.05$ compared with the respective patient group at each time point after surgery. Significant difference in pain score was noted between the LA and control groups, except at 6 h after surgery. All data were presented as mean ± SD. MBD: May be discharged.

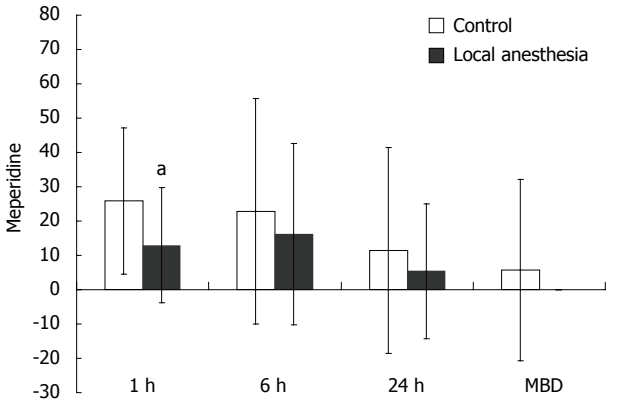

Figure 2 Meperidine required for LC patients in the LA and control groups. $^aP < 0.05$ when compared with the respective patient group at each time point after surgery, compared with the respective patient group before surgery. Significant difference was noted between the LA and control groups at 1 h after surgery. All data were presented as mean ± SD.

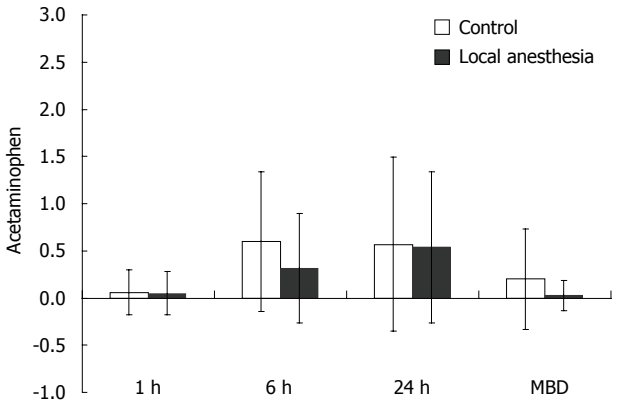

Figure 3 Acetaminophen required for LC patients in the LA and control groups. No significant difference was noted between the LA and control groups after surgery. All data were presented as mean ± SD.

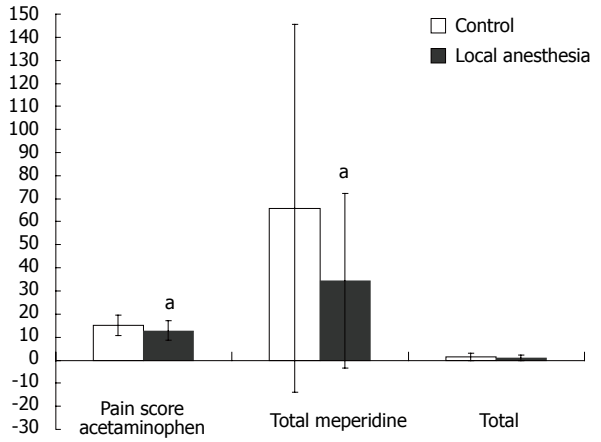

Figure 4 Pain evaluation and total analgesic requirement in LC patients in the LA and control groups. [a]*P* < 0.05 when compared with the respective patient group after surgery. Significant difference was noted for pain and meperidine requirement between the LA and control groups after surgery. All data were presented as mean ± SD.

early postoperative period after LC. This study clearly showed that pain reached a peak within the first few hours following the operation but diminished during the next 2-3 d, demonstrated by the distribution of the pain score and parenteral analgesic requirement. It has been reported that incisional pain is more intense than visceral pain and is dominant during the first 48 h after LC[13].

Several mechanisms have been proposed for generation of pain following laparoscopy: ruptured blood vessels caused by rapid distension of the peritoneum; traumatic traction on the nerves; release of inflammatory molecules; trauma to the abdominal wall, and when the gallbladder is removed from the abdomen; pneumoperitoneum created by use of CO_2; maintenance of high abdominal pressure; irritation of the phrenic nerve; and application of cold CO_2[14]. This explains why no consensus can be reached regarding effective postoperative pain relief in patients undergoing LC, because pain is multifactorial[11]. Although a number of studies have been conducted in an effort to reduce postoperative pain after surgery, the results have varied.

Postoperative pain control is directed at early mobilization, recovery, and discharge. However, pain also plays a major role in the metabolic and endocrine response, and is instrumental in the impairment of postoperative pulmonary function. Various methods have been investigated for reducing postoperative pain, such as local anesthesia[15], intraperitoneal infiltration of local anesthesia[16], preoperative administration of anti-inflammatory drugs[17], utilizing CO_2 at body temperature, applying intrapleural morphine[18], and combined use of NSAIDs and opioids[19].

Our findings indicated that infiltrating ropivacaine after surgery through the port site reduced pain intensity, the number of patients requiring postoperative analgesics, and hospital stay. Administering local anesthesia at the end of surgery offered a longer time delay to the need for analgesics, compared with patients who did not receive postoperative local anesthesia. Furthermore, patients who received local anesthesia at the end of surgery required significantly lower doses of analgesics than patients who did not receive local anesthesia. This is explained by the fact that pain intensity was less among patients who received local anesthesia at the end of the surgery than among those who did not.

Ropivacaine is a new long-acting local anesthetic that was developed after the emergence of bupivacaine-related severe toxicity. The agent is a pure left-isomer and, based on its three-dimensional structure, it has less toxic potential on the central nervous system and the heart[20]. Several clinical studies have evaluated its toxicology and clinical profiles: theoretically and experimentally, some differences can be seen, but reflection of these characteristics in clinical practice has not been evident. However, the reduced toxic potential of the pure left-isomer supports its use in clinical situations in which the risk of systemic toxicity related to overdosing or unwanted intravascular injection is high, such as during epidural or peripheral nerve blocks. Adverse effects associated with the use of local anesthesia, such as allergic reactions and local tissue, cardiovascular, central nervous system and systemic toxicity, were reported as rare in one previous study[20], and we did not observe any adverse effect related to the use of local anesthesia. Generally, the present study confirms earlier evidence that, in patients with gallbladder lesion undergoing LC, local anesthesia infusion is more effective when applied at the end of an operation than at the start.

Local anesthesia with ropivacaine infusion at the port site in LC patients at the end of surgery significantly decreased postoperative pain immediately. This short-term benefit explains the lower parenteral analgesic use and earlier discharge for LC patients with local anesthesia infusion. However, another clinical trial including multiple factors regarding pain after LC should be conducted.

COMMENTS

Background

Although laparoscopic cholecystectomy (LC) is a less painful procedure than open cholecystectomy, patients still felt wound pain after surgery. We tried to improve postoperative pain relief by the use of local anesthesia.

Research frontiers

Local anesthesia is used for postoperative analgesia and is effective. However, there have been few randomized studies performed. Good postoperative pain control will improve quality of life after LC.

Innovations and breakthroughs

Pain after LC may be caused by personal factors, duration of operation, intraperitoneal pressure, and the gallbladder lesion concerned. We used a prospective randomized trial to demonstrate that postoperative pain control improved by adding local anesthesia.

Applications

This local anesthesia procedure can be used routinely in other kinds of laparoscopic surgery to reduce postoperative pain.

Peer review

The authors reported that local anesthesia with ropivacaine infusion was beneficial for LC. The present study was concerned mainly with anesthesia. However, this paper is interesting and instructive for surgeons. The presentation and readability of the manuscript are good.

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Detection and evaluation of antibodies against neutrophil-activating protein of *Helicobacter pylori* in patients with gastric cancer

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Author contributions: Long M designed the study, analyzed and interpreted the data, and wrote the manuscript; Luo J cloned HP-NAP and detected its antibodies; Li Y cultured *Helicobacter pylori* and purified HP-NAP; Zeng FY collected the serum samples and supported the technology; Li M contributed to the acquisition funding and supervision.
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Abstract

AIM: To detect and evaluate the antibodies against *Helicobacter pylori* (*H pylori*) neutrophil-activating protein (HP-NAP) in patients with gastric cancer and other gastroduodenal diseases.

METHODS: Recombinant HP-NAP was prepared from a prokaryotic expression system in *Escherichia coli*. Serum positivity and level of HP-NAP-specific antibodies in sera from 43 patients with gastric cancer, 28 with chronic gastritis, 28 with peptic ulcer, and 89 healthy controls were measured by rHP-NAP-based ELISA. rHP-NAP-stimulated production of interleukin-8 (IL-8) and growth-related oncogene (GRO_α) cytokines in the culture supernatant of SGC7901 gastric epithelial cells was also detected.

RESULTS: The serum positivity and mean absorbance value of HP-NAP-specific antibodies in the gastric cancer group (97.7% and 1.01 ± 0.24) were significantly higher than those in the chronic gastritis group (85.7% and 0.89 ± 0.14, $P < 0.005$) and healthy control group (27.7% and 0.65 ± 0.18, $P < 0.001$). The sensitivity and specificity of ELISA for the detection of HP-NAP-specific antibodies were 95.5% and 91.5%, respectively. HP-NAP could slightly up-regulate IL-8 production in gastric epithelial cell lines but had no effect on GRO_α production.

CONCLUSION: Infection with virulent *H pylori* strains secreting HP-NAP is associated with severe gastroduodenal diseases, and HP-NAP may play a role in the development of gastric carcinoma. rHP-NAP-based ELISA can be used as a new method to detect *H pylori* infection. The direct effect of HP-NAP on gastric epithelial cells may be limited, but HP-NAP may contribute to inflammatory response or carcinogenesis by activating neutrophils.

Key words: *Helicobacter pylori*; *Helicobacter pylori* neutrophil-activating protein; Gastric cancer; Peptic ulcer; Chronic gastritis

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INTRODUCTION

Helicobacter pylori (*H pylori*), a microaerophilic Gram-negative bacterium, infects the stomach of more than 50% of human population worldwide and is a major cause of chronic gastritis and peptic ulcer. Furthermore, it is associated with gastric adenocarcinoma and gastric B cell lymphoma. In 1994, the World Health Organization classified *H pylori* infection as a definite (class 1) carcinogen[1]. *H pylori* colonization is followed by infiltration of neutrophils, macrophages and lymphocytes in gastric mucosa. The degree of mucosal damage is closely associated with the extent of

neutrophil infiltration[2-4].

Multiple bacterial virulence factors, such as vacA, cagA and lipopolysaccharide (LPS), can modulate *H pylori*-induced inflammation. *H pylori* neutrophil-activating protein (HP-NAP), a 150-kDa iron-binding protein, is a ball-shaped dodecamer formed by four-helix bundled subunits with its sequence similar to that of bacterioferritins and DNA binding proteins[5,6]. It has been designated as a neutrophil-activating factor because it promotes the adherence of neutrophils to endothelial cells and stimulates production of reactive oxygen species (ROS) in neutrophils by activating nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in plasma membrane[7-11]. Satin *et al*[12] demonstrated that the purified recombinant HP-NAP is chemotactic for human neutrophils and monocytes, induces surface expression of β_2-integrin, which mediates endothelial transmigration, adhesion and accumulation of leucocytes at the site of *H pylori* infection. Recombinant HP-NAP induces the production of ROS by neutrophils *via* a cascade of intracellular activation events, including increased cytosolic calcium and phosphorylation of proteins, leading to the assembly of functional NADPH oxidase on neutrophil plasma membrane. In addition, HP-NAP increases the synthesis of tissue factor and secretion of type 2 inhibitor of plasminogen activator in monocytes[13,14], contributing to the inflammation of gastric mucosa by fibrin deposition. These studies indicate that HP-NAP is a virulence factor relevant to the pathogenic effect of *H pylori*.

It was recently reported that HP-NAP promotes a Th1 immune response by inducing the expression of IL-12 and IL-23 in neutrophils and monocytes, and also elicits an antigen-specific Th1-polarized T cell response in gastric mucosa of *H pylori*-infected patients *in vivo*[15]. It has been shown that HP-NAP is able to shift antigen-activated human T cells from a Th2 to a Th1 cytotoxic phenotype characterized by production of IFN-γ and TNF-α[16]. Additionally, the majority of infected patients have antibodies against this antigen, and vaccination of mice with HP-NAP induces protection against a subsequent challenge with *H pylori*[12]. Therefore, HP-NAP is an immune modulator promoting Th1 immune responses and an important vaccine candidate antigen[17,18].

Since HP-NAP is a powerful stimulant for the production of ROS, mediating damage to DNA and enhancing cell turnover[19], it may be a risk factor for *H pylori*-associated gastric cancer. Currently, it is uncertain whether HP-NAP is related to the occurrence of gastric cancer. Interleukin-8 (IL-8) and growth-related oncogene (GRO_α) are members of the CXC chemokine family that induce neutrophil chemotaxis and activation. It has been shown that IL-8 and GRO_α levels are elevated in *H pylori*-infected gastric mucosa[20]. In addition, *H pylori* water soluble surface proteins up-regulate the expression of IL-8 and GRO_α mRNA and protein by neutrophils[21]. Whether HP-NAP contributes to the inflammatory response or carcinogenesis by up-regulating IL-8 and GRO_α production in *H pylori*-infected gastric mucosa remains unknown. An understanding of the relation between HP-NAP and gastric cancer and the molecular mechanism(s) underlying HP-NAP-induced diseases should lead to improved approaches to the effective control of *H pylori*-associated gastric cancer.

In the present study, recombinant HP-NAP was prepared from a prokaryotic expression system in *Escherichia coli*, and the *napA* gene in 20 *H pylori* clinical isolates from South China was detected by PCR. The seropositivity and level of HP-NAP-specific antibodies in sera from 43 patients with gastric cancer, 28 with chronic gastritis, 28 with peptic ulcer, and 89 healthy controls were measured by rHP-NAP-based ELISA. The production of IL-8 and GRO_α cytokines in culture supernatant from SGC7901 gastric epithelial cells stimulated by rHP-NAP was also detected.

MATERIALS AND METHODS

Preparation of bacterial and gastric epithelial cell lines

H pylori NCTC11639 strain was stored at -70℃ in our department. Bacteria were routinely cultured on Columbia agar plates supplemented with 10% defibrinated sheep blood, 0.004% triphenyltetrazolium chloride, and Dent selective supplement (Oxoid, Basingstoke, UK) at 37℃ for 3 d under a microaerophilic atmosphere containing 50 mL/L O_2, 150 mL/L CO_2 and 800 mL/L N_2. Several colonies were then picked up and inoculated into 20 mL of Brucella broth (Becton Dickinson, Cockeysville, MS) containing 0.1% β-cyclodextrin supplemented with 5% (v/v) fetal calf serum. After 24 h, 2 mL of culture was transferred to 40 mL of fresh medium, and the same process was repeated twice. Finally, 1 mL of the incubated medium containing the bacterial cells, most of which were spiral rather than coccoid, was plated on Brucella agar (Becton Dickinson) containing 10% (v/v) defibrinated sheep blood and cultured at 37℃ for an additional 3 d in a microaerophilic atmosphere containing 50 mL/L O_2, 150 mL/L CO_2 and 800 mL/L N_2. Bacterial cells were harvested, washed twice with cold phosphate-buffered saline (PBS, 25 mmol/L sodium phosphate, pH 7.2, 0.9% NaCl), and then sedimented by centrifugation at 5000 × *g* for 10 min at 4℃. The cell pellet was stored at -80℃.

Human gastric epithelial cells (SGC7901) were cultured at 37℃ in RPMI-1640 (Gibco, USA) containing 10% FBS (Gibco) in a humidified atmosphere containing 50 mL/L CO_2, and plated at 10^6 cells/well in 24-well plates. The medium was changed every 3 d and replaced with RPMI-1640 without serum before experiment.

Collection of serum samples from infected and healthy individuals

H pylori infection was diagnosed by histological examination of endoscopic biopsy specimens and CLO testing. Forty-three serum samples were collected from patients with gastric cancer at Southern Hospital, Guangzhou, China. The age of patients ranged 27-83 years. Twenty-eight serum samples were also collected from patients with peptic ulcer or chronic gastritis at Southern Hospital. The age of patients ranged 28-67 years. Finally, 89 serum samples were collected from healthy blood donors at the age of 18-70 years.

Cloning and purification of NAP

Genomic DNA of *H pylori* was prepared using a Takara kit (Takara, Japan) according to its manufacturer's instructions. The extracted genomic DNA was then used as a template for amplification of the NAP coding region using a Taq DNA polymerase PCR kit (Takara, Japan)[22,23]. Two primer sequences corresponding to the 5' and 3' ends of the coding gene, including *Eco*RI and *Xho*I restriction sites, were used: P1: 5'CCGGAATTCA TGAAAACATTTGAA-3', P2: 5'CCGCTCGAGTTAA GCCAAATGGGC-3'.

The PCR product was cloned into the expression vector pGEX-4T-1 (Amersham Biosciences). The plasmid was then transformed into *E. coli* strain TOP10 (Invitrogen BV, Leek, The Netherlands), and NAP expression was induced with 1 mmol/L isopropyl-β-d-1-thiogalactopyranoside when the cells were grown to the log phase at room temperature. After 4 h, the cells were harvested by centrifugation and washed with ice-cold PBS containing 5 mmol/L EDTA and 2 mmol/L PMSF. All subsequent procedures were performed at 4℃. The NAP-GST fusion protein was purified by glutathione-sepharose 4B column chromatography.

Screening for seropositive individuals with IgG antibodies against *H pylori* in healthy subjects

Anti-*H pylori* IgG antibodies in serum samples were assayed by indirect ELISA using a diagnostic kit (BIOCUP, Shenzhen, China). According to the instructions, serum (l00 μL, diluted 1/100 in PBS) was added to ELISA plates pre-coated with purified *H pylori* antigens in duplicate and incubated for 1 h. Controls consisted of wells with PBS alone, *H pylori* negative and positive serum, which were considered blank, negative and positive controls, respectively. Peroxidase-conjugated anti-human IgG (1/5000) was added and incubated for 30 min, after which the plates were washed with PBS, and color reaction was initiated by the addition of TMB (100 μL). After 10 min, the reaction was terminated by the addition of 1 mol/L H_2SO_4 (100 μL). The plate reader was calibrated to the blank well and the absorbance at 450 nm was read. Cut-off value (C.O.) = 2.1 × Nc (Nc = the mean absorbance value for three negative controls). Samples with absorbance > 0.21 were considered positive. The anti-*H pylori* IgG seropositive individuals were considered to be *H pylori*-infected healthy individuals.

Detection of antibodies against HP-NAP in serum by ELISA

Recombinant HP-NAP was prepared and purified, and ELISA was carried out as previously described[24-27]. Briefly, immunoplates (Nunc, Denmark) were coated with rNAP (5μg/well) and incubated overnight at 4℃. After washed three times with a washing buffer containing PBS (pH 7.2) and 0.1% Tween 20, the plates were blocked with 200 μL of 10% bovine serum in PBS and incubated in a moist chamber for 1 h at 37℃, then washed three times with a washing buffer containing PBS (pH 7.2) and 0.1% Tween 20. One hundred microlitre of serum samples (1:100) from patients or healthy individuals was then added to the microtiter wells, and the plates were incubated at 37℃ for 60 min. After washed with PBS, 100 μL of secondary antibody (goat anti-human IgG-HRP, 1:10 000) was added to each well and incubated for 60 min at 37℃. After washed with PBS, 100 μL of TMB/H_2O_2 substrate was added to the wells and incubated at room temperature for about 10 min. The reaction was terminated by adding 100 μL of 2 mol/L H_2SO_4. The absorbance of each well was read at 450 nm. Samples with absorbance > 0.78 were considered positive. Each sample was tested in duplicate.

Selection of cut-off values for antibodies against HP-NAP in human serum

A receiver operating curve (ROC) was plotted to calculate the cut-off values for antibodies against HP-NAP at a 95% accuracy level[26]. In addition, the cut-off values were determined by mean plus 2 × SD and mean plus 3 × SD, derived from *P* values in healthy individuals.

Detection of the *napA* gene in *H pylori* clinical isolates by PCR

Genomic DNA of 20 *H pylori* clinical isolates was prepared and used as a template for amplification of the NAP coding region with two primer sequences as preciously described[28]. PCR was carried out in a final volume of 60 μL. A preliminary denaturation step at 95℃ for 5 min was followed by 30 amplification cycles, each consisting of denaturation at 94℃ for 30 s, annealing at 50℃ for 30 s, extension at 72℃ for 45 s, and a final extension at 72℃ for 7 min. Then, 10 μL of amplicon was transferred onto a 1.5% (w/v) agarose gel in 1 × TBE buffer, and electrophoresis was performed at 80 V for 30 min in a DNA submarine plate. The gel was then stained with ethidium bromide.

IL-8 and GRO$_\alpha$ production in human gastric epithelial cells stimulated with rHP-NAP

Before use, human gastric epithelial cells (SGC7901) growing in 24-well plates were washed and replaced with RPMI-1640 without serum, and then stimulated with 10 μg rHP-NAP for 12 h and 36 h, respectively. The supernatant was centrifuged to remove particulate debris and stored in aliquots at -70℃. IL-8 and GRO$_\alpha$ concentrations in the culture supernatant were measured by ELISA using a Quantikine immunoassay kit (R&D, USA) according to its manufacturer's instructions. Before detection, the culture supernatant was filtered through a sterile membrane filter (0.22 μm. pore size).

Statistical analysis

Statistical analyses were conducted using the SPSS software package. One-way ANOVA was performed to assess differences among groups. *Post hoc* multiple comparisons between different infectious groups were done by LSD analysis. Comparisons between the seropositivity of anti-HP-NAP antibodies in patients with gastric cancer and other gastroduodenal diseases were done by chi-square test.

RESULTS

Expression and purification of recombinant protein

The gene encoding for HP-NAP was obtained by PCR amplification using genomic DNA extracted from *H pylori* as a template. Agarose gel electrophoresis analysis of the PCR product is shown in Figure 1.

A 435 bp DNA fragment was detected, which corresponded to the size of the HP-NAP gene. The DNA sequence of the hypothetical HP-NAP gene of *H pylori* was determined (data not shown) and submitted to GenBank (accession No. DQ341279). To facilitate purification, HP-NAP was expressed as a GST fusion protein in *E. coli* Top10 cells. Expression of the recombinant NAP-GST was examined by SDS-PAGE. A pronounced band with an approximate molecular weight of 44 kDa appeared in the supernatant of cell lysate after induction but not in control cells, suggesting that the fusion protein can be successfully expressed in bacterial cells (Figure 2).

The purified NAP-GST fusion protein was further confirmed by Western blotting. Serum from *H pylori*-infected patients specifically recognized the recombinant NAP-GST fusion protein, while negative serum did not (Figure 3).

Selection of cut-off values for HP-NAP-specific antibodies

A cut-off value of 0.78 was determined for ELISA of HP-NAP antibodies using ROC analysis (Figure 4). The area under the ROC curve was 0.97. Overall, ELISA yielded a sensitivity of 95.5% (95% confidence interval) and a specificity of 91.5% (95% confidence interval) for the detection of antibodies against HP-NAP in serum.

Comparison between seropositivity for anti-*H pylori* IgG and anti-HP-NAP antibodies in healthy subjects

Anti-*H pylori* IgG seropositivity was detected in 47 of the 89 healthy subjects who were considered *H pylori*-infected healthy individuals. As shown in Table 1, the anti-*H pylori* IgG and anti-HP-NAP seroposivity in healthy individuals was 52.8% (47/89) and 14.6% (13/89), respectively. The anti-*H pylori* IgG seropositivity was much higher than the anti-HP-NAP antibody seropositivity ($P < 0.005$). The anti-HP-NAP antibody seropositivity, however, was 27.7% (13/47) in *H pylori*-infected healthy individuals.

Detection of antibodies against HP-NAP in patients with gastric cancer and other gastroduodenal diseases by ELISA

The seropositivity for antibodies against HP-NAP in gastric cancer and peptic ulcer patients was 97.7% (42/43) and 92.8% (26/28), respectively, while the seropositivity for antibodies against HP-NAP in chronic gastritis patients was 85.7% (24/28). The seropositivity for antibodies against HP-NAP in infected healthy controls was 27.7%. The seropositivity for HP-NAP-specific antibodies in gastric cancer patients was higher than that in chronic gastritis patients ($P < 0.05$) and in infected healthy controls ($P < 0.01$). The difference in the mean absorbance value for antibodies against HP-NAP between groups was also confirmed by one-way

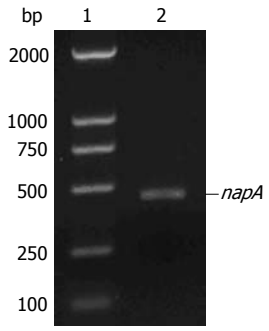

Figure 1 Agarose gel electrophoresis of *napA* gene amplified by PCR. Lane 1: DL2000 DNA marker; lane 2: *H pylori napA* gene (435 bp).

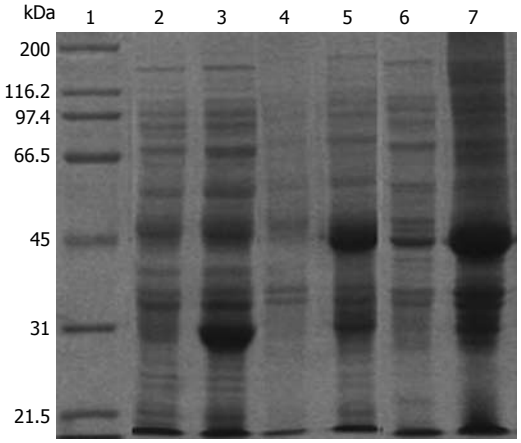

Figure 2 SDS-PAGE analysis of the expression of pGEX-4T-1/HP-NAP in *E. coli*. Lane 1: Protein marker; Lane 2: pGEX-4T-1 before induction; Lane 3: pGEX-4T-1 after induction; Lane 4: pGEX-4T-1/HP-NAP before induction; Lane 5: pGEX-4T-1/HP-NAP after induction; Lane 6: Supernatant of *E. coli* after induction; Lane 7: Lysate of *E. coli* after induction.

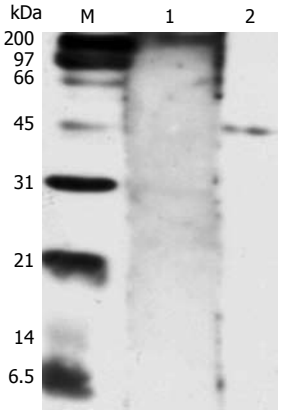

Figure 3 Western blotting analysis of HP-NAP reactivity with the positive serum from *H pylori*-infected patients. Lane 1: Serum from a control patient not infected with *H pylori*; Lane 2: Serum from a patient infected with *H pylori*; M: Protein marker.

Table 1 Positivity comparison between *H pylori* -specific IgG and HP-NAP-specific antibodies in healthy individuals *n* (%)

	Total number tested	Positivity
HP-specific IgG Abs	89	47 (52.8)
HP-NAP-specific Abs (total healthy persons)	89	13 (14.6)[1]
HP-NAP-specific Abs (infected healthy persons)	47	13 (27.7)

[1]$P < 0.005$ *vs* infected healthy persons; Abs: Antibodies.

Table 2 Demonstration of antibodies against HP-NAP in serum from *H pylori*-infected patients by ELISA *n* (%)

Patient group	Positivity for HP-NAP antibodies	Negativity for HP-NAP antibodies	Absorbance (mean ± 2SD) (cut-off value 0.78)	Range
Gastric cancer group (n = 43)	42 (97.7)	1 (2.3)	1.01 ± 0.24	0.748-1.269
Peptic ulcer group (n = 28)	26 (92.8)	2 (7.1)	0.98 ± 0.32	0.771-1.265
Chronic gastritis group (n = 28)	24 (85.7)	4 (14.3)	0.89 ± 0.14	0.711-1.122
Healthy persons (HP-IgG positive n = 47)	13 (27.7)	34 (72.3)	0.65 ± 0.18	0.451-0.948

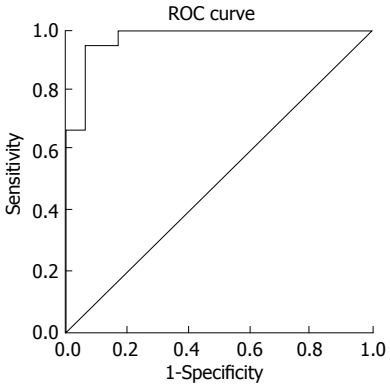

Figure 4 Receiver operating characteristic curve analysis showing the cut-off value for anti-HP-NAP antibodies in serum from patients with gastric cancer, peptic ulcer, chronic gastritis and healthy controls, respectively.

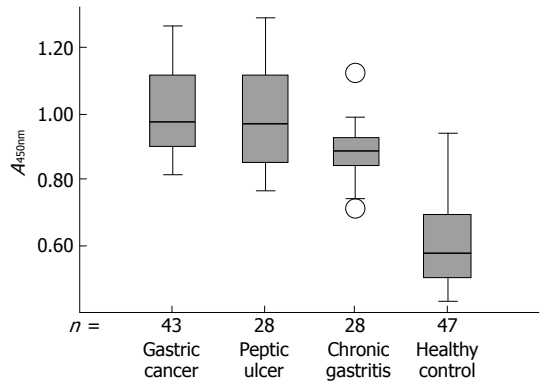

Figure 5 Box plot demonstrating the level of antibodies against HP-NAP in serum from patients with gastric cancer, peptic ulcer, chronic gastritis and healthy controls, respectively. The box plot showing the 5th and 95th percentiles (bars), the 75th and 25th percentiles (boxes), and the median (bars in boxes), respectively. *n*: Number of individuals in each group.

ANOVA analysis (F = 4.014, P = 0.023). The mean absorbance value for anti-HP-NAP antibodies in gastric cancer patients was 1.01 ± 0.24 (range 0.748-1.269), significantly higher than that in chronic gastritis patients (0.89 ± 0.14, range 0.711-1.122, $P < 0.005$) and in infected healthy controls (0.65 ± 0.18, range 0.451-0.948, $P < 0.001$). There was no significant difference, however, in the mean absorbance value for anti-HP-NAP antibodies between patients with gastric cancer (1.01 ± 0.24, range 0.748-1.269) and peptic ulcer (0.98 ± 0.32, range 0.771-1.265) (Table 2).

Box plots of the antibodies against HP-NAP in sera from patients with gastric cancer, peptic ulcer, chronic gastritis, and healthy controls are shown in Figure 5, with the 90th percentile range and 75th and 25th percentiles.

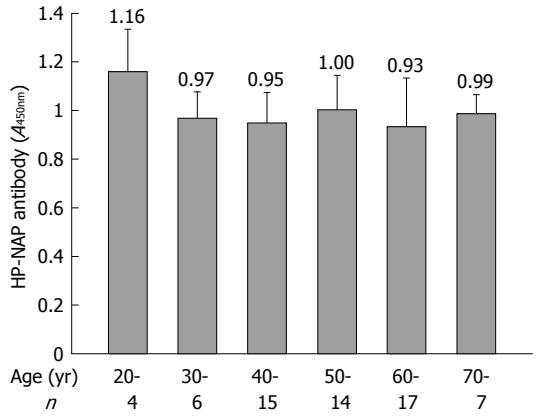

Figure 6 Comparison of HP-NAP antibody levels in different age groups.

Comparison of HP-NAP antibody levels in different age groups

Most *H pylori*-associated diseases occurred in subjects at the ages of 40-70 years (53/63). The mean absorbance value for anti-HP-NAP antibodies was not significantly different in patients at different ages (Figure 6).

Detection of napA gene in all H pylori clinical isolates

The *napA* gene was detected by PCR in 20 *H pylori* clinical isolates. All examined *H pylori* strains carried the *napA* gene. The electrophoresis results of PCR products of the *napA* gene from 8 representative *H pylori* strains are shown in Figure 7.

IL-8 and GROα production in human gastric epithelial cells stimulated with rHP-NAP

The IL-8 and GROα levels in culture supernatant of human gastric epithelial cell line SGC7901 were measured by ELISA, showing that HP-NAP slightly up-regulated the IL-8 production (Table 3) but had no effect on GROα production by gastric epithelial cells (data not shown).

DISCUSSION

HP-NAP, an important virulence factor for *H pylori*, induces adherence of neutrophils to gastric epithelial cells and causes an inflammatory reaction by activating neutrophils. In the present study, we cloned and expressed HP-NAP, which was then used as an antigen to detect HP-NAP-specific antibodies in serum samples. The seropositivity for anti-HP-NAP antibodies was detected in 89 healthy subjects and compared with that for anti-*H pylori* IgG antibodies in the same subjects. Meanwhile,

Table 3 IL-8 level in culture supernatant of human gastric epithelial cell line SCG7901 (pg/mL)

Time (h)	IL-8 level		
	10 μg/mL NAP	1 μg/mL LPS	Control
12	10.10[b]	8.07	3.55
36	30.14[b]	30.54	11.28

[b]$P < 0.01$ *vs* control.

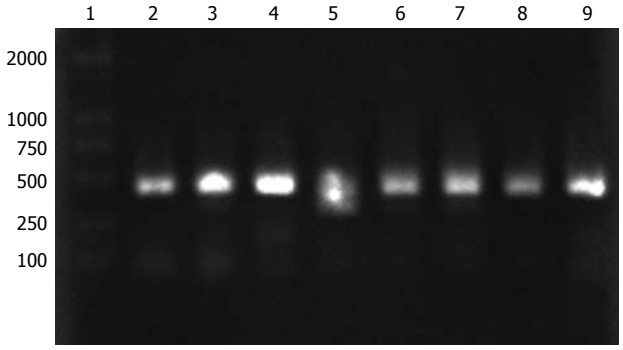

Figure 7 Agarose gel electrophoresis analysis of PCR product of *napA* gene from *H pylori* clinic strains. Lane 1: DL2000 DNA marker; Lanes 2-9: *napA* gene (435 bp) from eight *H pylori* clinic strains.

the seropositivity and levels of HP-NAP-specific antibodies in 43 patients with gastric cancer, 28 with chronic gastritis, 28 with peptic ulcer, were measured by rHP-NAP-based ELISA. Moreover, the *napA* gene in 20 *H pylori* clinical isolates from South China was detected by PCR. The production of IL-8 and GROα cytokines in the culture supernatant of SGC7901 gastric epithelial cells stimulated with rHP-NAP was also detected.

The seropositivity for anti-*H pylori* IgG was 52.8% (47/89) in healthy subjects, which is in agreement with the average prevalence (about 50%-60%) of *H pylori* in the adult Chinese population. The 47 subjects who were serum positive for anti-*H pylori* IgG were considered to be *H pylori*-infected healthy individuals. The seropositivity for anti-HP-NAP antibodies was 27.7% (13/47) in *H pylori*-infected healthy individuals, due to variations in HP-NAP expression in different *H pylori* strains. It has been shown that the neutrophil adhesion-promoting activity in different *H pylori* strains varies considerably[29,30], suggesting that HP-NAP is differently expressed in different *H pylori* strains[31]. Our results also show that the mean absorbance value for anti-HP-NAP antibodies was not significantly different in different age groups. Our work and other studies, however, have found that the *napA* gene is present in all *H pylori* clinical isolates.

H pylori is a clear (class 1) carcinogen. HP-NAP stimulates neutrophils to infiltrate gastric mucosa and subsequently causes ROS production by activating NADPH oxidase in plasma membrane. It was reported that ROS can cause a variety of DNA lesions and produce mutations in mammalian cells[32]. ROS production is significantly decreased in gastric mucosa of patients with *H pylori* successfully eradicated[33]. It has also been shown that HP-NAP can be involved in extravasation of leukocytes, and ROS can play a role in the carcinogenic process in gastric mucosa during chronic *H pylori* infection[34,35], indicating that HP-NAP may be a risk factor for *H pylori*-associated gastric cancer.

In the present study, the seropositivity and mean absorbance value for HP-NAP-specific antibodies in gastric cancer patients (97.7% and 1.01 ± 0.24) were significantly higher than those in chronic gastritis patients (85.7% and 0.89 ± 0.14, $P < 0.005$) and in infected healthy controls (0.65 ± 0.18, range 0.451-0.948, $P < 0.001$). There was no difference, however, in the seropositivity or mean absorbance value for HP-NAP antibodies between the patients with gastric cancer and peptic ulcer, indicating that HP-NAP specific antibodies are correlated with severe gastroduodenal diseases and HP-NAP may contribute to the pathogenesis of *H pylori*-associated gastric cancer. Our results also show that HP-NAP had a strong antigenicity, and the majority of infected patients produced antibodies against HP-NAP.

ELISA for the detection of antibodies against HP-NAP in this study had a sensitivity of 95.5% and a specificity of 91.5%. Since the level of HP-NAP-specific antibodies may be correlated with the severity of *H pylori* infection, ELISA can be used in immunodiagnostic assay for *H pylori*-infection and in screening for a high-risk population with *H pylori*-associated gastric cancer.

In this study, HP-NAP slightly up-regulated IL-8 production by gastric epithelial cell lines, but had no effect on GROα production, suggesting that the direct effect of HP-NAP on gastric epithelial cells may be limited, but HP-NAP may contribute to the inflammatory response or carcinogenesis by activating neutrophils.

In conclusion, infection with virulent *H pylori* strains secreting HP-NAP is associated with severe gastroduodenal diseases and HP-NAP may play a role in the development of gastric carcinoma. rHP-NAP-based ELISA can be used as a new method to detect *H pylori* infection. We have recently developed a monoclonal antibody against HP-NAP, which might be used to detect HP-NAP expression in gastric mucosa. It would be desirable to carry out a comparative analysis to define the characteristic differences in HP-NAP expression between patients with gastric cancer and other gastroduodenal diseases. Further characterization of the interactions between HP-NAP and gastric cancer should aid in the development of novel strategies against *H pylori*-associated gastric cancer.

COMMENTS

Background

Helicobacter pylori (H pylori) infection increases the risk of developing peptic ulcer, gastric adenocarcinoma, and gastric B cell lymphoma. *H pylori* neutrophil-activating protein (HP-NAP), a virulence factor, promotes the adherence of neutrophils to gastric mucosa endothelial cells and stimulates high production of reactive oxygen species (ROS) in neutrophils. Since ROS can mediate DNA damage and enhance cell turnover, HP-NAP may be a risk factor for *H pylori*-associated gastric cancer. To prevent *H pylori*-related cancer, mass screening for and treatment of *H pylori* infection are cost-effective. Whether HP-NAP is related to the occurrence of gastric cancer is still uncertain.

Research frontiers

HP-NAP is a virulence factor for *H pylori* infection and a vaccine candidate antigen. The majority of infected patients have antibodies against this antigen,

and vaccination of HP-NAP in mice can protect against a subsequent challenge with *H pylori*. *H pylori* colonization is followed by infiltration of neutrophils, macrophages and lymphocytes in gastric mucosa. The degree of mucosal damage is closely correlated with the extent of neutrophil infiltration. It has been shown that HP-NAP can be involved in the extravasation of leukocytes, and ROS can play a role in the carcinogenic process of gastric mucosa during chronic *H pylori* infection.

Innovations and breakthroughs

An understanding of the relation between HP-NAP and gastric cancer should lead to improved approaches to the effective control of *H pylori*-associated gastric cancer. Seropositivity and mean absorbance value for HP-NAP-specific antibodies in gastric cancer patients were significantly higher than those in chronic gastritis patients and in infected healthy controls. There was no difference, however, in serum positivity or mean absorbance value for HP-NAP antibodies between patients with gastric cancer and peptic ulcer. These findings indicate that HP-NAP specific antibodies are correlated with severe gastroduodenal diseases and HP-NAP may contribute to the pathogenesis of *H pylori*-associated stomach cancer.

Applications

ELISA used for the detection of antibodies against HP-NAP in this study had a sensitivity of 95.5% and a specificity of 91.5%. Since the level of HP-NAP-specific antibodies may be correlated with the severity of *H pylori* infection, ELISA can be used to screen for a high-risk population with *H pylori*-associated gastric cancer.

Terminology

H pylori, a gram-negative microaerophilic bacterium, causes a long-term mild inflammation of stomach lining and is strongly linked to the development of duodenal and gastric ulcers and stomach cancer. Over 50% of the world's population harbor *H pylori* in their upper gastrointestinal tract, and infection is more prevalent in developing countries.

Peer review

In this study, the authors detected and evaluated the level of antibodies against HP-NAP in patients with gastric cancer and other gastroduodenal diseases. The results suggest that HP-NAP-specific antibodies are correlated with severe gastroduodenal diseases and HP-NAP may contribute to the pathogenesis of *H pylori*-associated stomach cancer. ELISA can be used to screen for a high-risk population with *H pylori*-associated gastric cancer.

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Association between Bmi1 and clinicopathological status of esophageal squamous cell carcinoma

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Abstract

AIM: To investigate the clinicopathological roles of Bmi1 in esophageal squamous cell carcinoma (ESCC).

METHODS: Quantitative real-time polymerase chain reaction and immunohistochemical staining for Bmi1 were performed in cancerous and adjacent non-cancerous paraffin-embedded esophageal specimens.

RESULTS: The Bmi1 expression level was unaffected by gender and age. The level of Bmi1 mRNA in ESCC was significantly higher than that in the adjacent non-cancerous tissues (2.181 ± 2.158 *vs* 0.931 ± 0.894, $P = 0.0152$), and its over-expression was aggressively associated with lymph node metastasis (3.580 ± 2.487 *vs* 1.703 ± 0.758, $P = 0.0003$), poorer cell differentiation ($P = 0.0000$) and advanced pathological stage (3.827 ± 2.673 *vs* 1.590 ± 0.735, $P = 0.0001$). The patients were divided into high-expression and low-expression groups based on the median expression level of Bmi1 mRNA, and a shorter overall survival time in the former group was observed. Immunohistochemistry for Bmi1 oncoprotein showed diffusely positive, focally positive and negative expression in 44, 16 and 10 of 70 ESCC cases, respectively, compared with three, two and five of 10 adjacent non-cancerous cases ($P = 0.027$). The positive rate of the oncoprotein in samples of histological grade Ⅲ was higher than that of grade Ⅱ ($P = 0.031$), but its expression had no relation to the lymph node metastasis and pathological staging. In 70 ESCC samples, Bmi1 showed high intense expression in the cytoplasm and less or even no expression in the nucleus.

CONCLUSION: Bmi1 was over-expressed in ESCC. Increased Bmi1 mRNA expression was significantly associated with ESCC progression, and the oncoprotein was largely distributed in the cytoplasm of tumor cells.

Key words: Esophageal squamous cell carcinoma; Bmi1; Quantitative real-time polymerase chain reaction; Immunohistochemistry; Clinicopathology

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INTRODUCTION

Esophageal cancer is one of the most frequently occurring malignancies and the seventh leading cause of cancer-related deaths in the world. It exhibits considerable geographic variation, and 95% of tumors are esophageal squamous cell carcinoma (ESCC)[1]. Besides the impact of the environment, the process of esophageal tumorigenesis at the molecular level is related to disorders of cell amplification, differentiation, senescence and apoptosis. The genetic bases underlying esophageal tumorigenesis have been partly understood in the past few years, including a loss of the anti-oncogene p53 and over-expression of epidermal growth

factor receptor or c-Myc[2]. However, other molecular mechanisms involved in esophageal tumorigenesis progress are still largely unknown.

Bmi1, located in 10p11.23, is a member of the polycomb group (PcG) and a component of the polycomb repressive complex 1. It was initially identified as an oncogene cooperating with c-Myc in the generation of lymphomas in double transgenic mice[3-5]. Several lines of evidence imply that Bmi1 plays an important role in the regulation of cell proliferation and senescence and is required for maintenance of adult hematopoietic and neural stem cells[6-9]. *Bmi1* gene amplification is observed mainly in mantle cell lymphomas[10], and recent serial studies have shown that Bmi1 is overexpressed in many somatic solid tumors such as colon carcinoma, non-small cell lung cancer, breast cancer, head and neck squamous cell carcinoma and gastric carcinoma[11-15], and it may be of diagnostic and prognostic relevance. However, to date, no report about the role of Bmi1 in ESCC has been made. The up-regulation of c-Myc and the down-regulation of p53 and p16 in ESCC[2] tissues make it plausible that Bmi1 may play an important role in the initiation and development of ESCC. This study was designed to investigate Bmi1 expression in ESCC tissues and its impact on patients with ESCC.

MATERIALS AND METHODS

Ethics

The use of study specimens for analyses was approved by the Research Ethics Committee of Nanjing Medical University. Informed written consent was obtained from all the patients.

Case selection

From June 1997 to February 2000, 80 ESCC and 15 adjacent non-cancerous paraffin-embedded samples were obtained from the tumor center of Nanjing First Hospital affiliated to Nanjing Medical University. There were 52 male and 28 female patients with a mean age of 60 years (range: 41-82). The patients were given preoperative examination including biopsy for diagnosis, barium X-ray, CT and ultrasonic endoscopy for clinical staging, and no treatment was given before operation. Radical resection was performed in each patient, and all the samples underwent postoperative pathological examination. There were 54 cases of stage Ⅰ-Ⅱ and 26 cases of stage Ⅲ-Ⅳ cancer according to the American Joint Committee on Cancer staging manual (AJCC, 2002)[16]. With regard to postoperative histological results, 16 were well-differentiated, 40 moderately differentiated and 24 poorly differentiated. Another 70 ESCC and 10 non-cancerous paraffin-embedded samples were enlisted from January 2002 to December 2003 in the same institution. There were 48 male and 22 female patients with a mean age of 61 years (range: 38-89). All the patients were assessed for physiological ability and endoscopy and CT scan were performed for clinical staging prior to routine surgery for ESCC. The postoperative pathological examination found 56 cases of stage Ⅰ-Ⅱ and 14 cases of Ⅲ-Ⅳ cancer according to AJCC (2002) pTNM standards[16]. Clinical follow-up after surgery and diagnosis was based on periodic visits (every 3 mo during the first year, every 6 mo the second year, and then yearly until relapse).

RNA extraction and quantitative real-time polymerase chain reaction (PCR)

Real-time quantitative PCR was performed on paraffin-embedded sections from 80 ESCC patients and 15 adjacent non-cancerous samples. Briefly, total RNA was extracted by Recover All Total Nucleic Acid Isolation kit (Ambion), and 10 mg of DNase-treated total RNA was used for reverse transcription with Superscript Ⅲ (Invitrogen, Carlsbad, CA, USA). An aliquot representing 100 ng input RNA was amplified by quantitative real-time PCR using the TaqMan PCR reagent kit and assay-on-demand gene expression products (FAM/Sybr, Foster City, CA, USA). RNA extracted from a non-cancerous lesion in one patient was used as a standard. After reverse transcription, standard cDNA was serially diluted to obtain five standard solutions for use in PCR to generate the reference curve. Sequences of the Bmi1 bidirectional primers were designed using Primer 5.0 rotor-gene 6.0 (Corbett Research) as follows: Bmi1 sense 5'-GTATTCCC TCCACCTCTTCTTG-3', Bmi1 antisense 5'-TGCTGAT GACCCATTTACTGAT-3'. House-keeping gene: β-actin sense 5'-CCTGTACGCCAACACAGTGC-3', antisense 5'-ATACTCCTGCTTGCTGATCC-3'. Quantitative real-time PCR was carried out in a Rotor-Gene 3000 PCR kit (Corbett Research) with 10000 × Syber Green (Molecular Probes). After reverse transcription, standard cDNA was serially diluted to obtain five standard solutions for use in PCR to generate the reference curve. The relative amount of cDNA in each sample was measured by interpolation using the standard curve (Figure 1), and then the relative ratio of Bmi1 to β-actin (housekeeping gene) expression was calculated for each ESCC sample.

Immunohistochemistry

Histopathological evaluation was performed on 4-μm slides stained with hematoxylin and eosin (HE) (Figure 2). Commercially available rabbit monoclonal antibodies against Bmi-1 (1:100, Santa Cruz Biotechnology) were used as primary antibodies. A paraffin section of the ESCC sample was deparaffinized and rehydrated in graded alcohol to water. Antigenic enhancement was performed by submerging in citrate buffer (pH 6.0) and microwaving. Endogenous peroxide activity was quenched by applying 0.3% hydrogen peroxide for 10 min, followed by incubation with 1% BSA to block the non-specific binding. The primary monoclonal anti-Bmi1 antibody was incubated for 60 min at 37℃. After washing, the tissue section was reacted with the biotinylated anti-rabbit IgG, and visualized using a Dako Envision System horseradish peroxidase for monoclonal antibodies. The slides were immersed in the prepared diaminobenzidine solution, which produces a brown precipitate at the level of the antigen-primary antibody. Slides were then counterstained with hematoxylin, dehydrated through alcohols of increasing concentration, placed in xylene, coverslipped using Permount, and

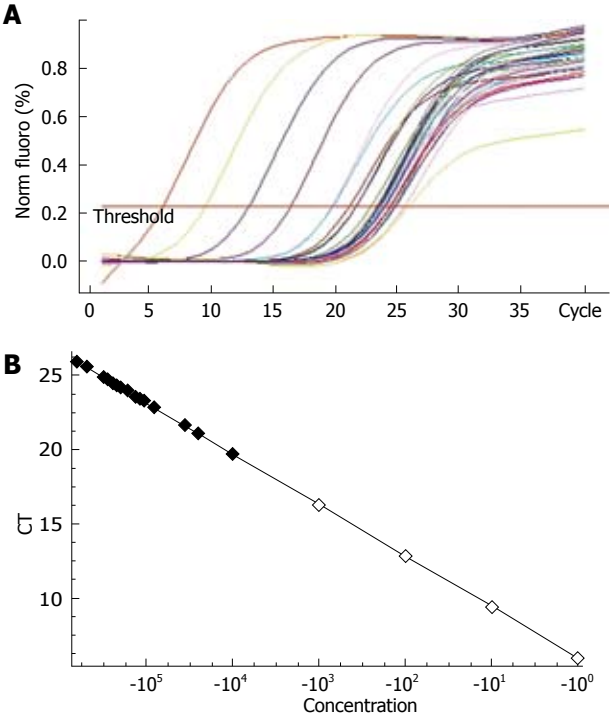

Figure 1 Amplification curve (A) and standard curve (B) of quantitative real-time PCR for Bmi1 mRNA. Total RNA was extracted for subsequent reverse transcription, and standard cDNA was serially diluted to obtain five standard solutions (1×10^{-1}, 1×10^{-2}, 1×10^{-3}, 1×10^{-4}, 1×10^{-5}) for use in PCR to generate the reference curve, slope rate of the straight line (a) = -3.42089, intercept (b) = 5.97517, correlation coefficient (*r*) = 0.9996. The strength of Bmi1 and β-actin was directly generated by the machine.

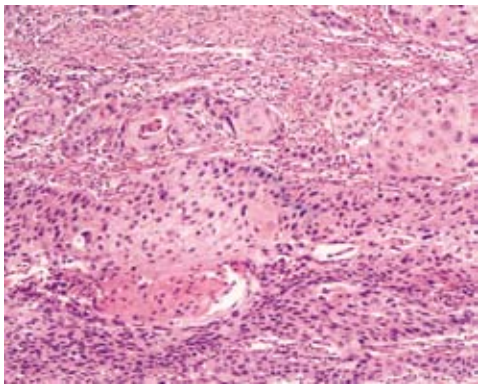

Figure 2 Staining of ESCC tissues. The tumor cells of cancerous tissues were stained as violet in the nucleus and pink in the cytoplasm (× 100).

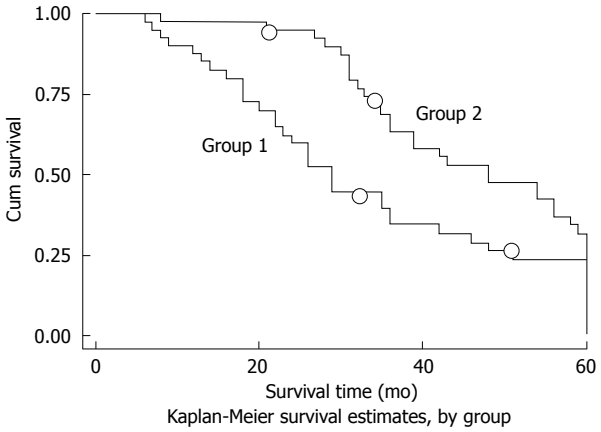

Figure 3 Effects of Bmi1 mRNA expression on prognosis of ESCC patients. Two patients did not complete the 5-year follow-up in each group (small circles). The survival rate was higher in the down-expression group (group 2) than in the up-expression group (group 1), $\chi^2 = 4.41$, $P = 0.0356$.

analyzed under light microscopy. Each section was evaluated by at least two independent professional pathologists, the distribution of Bmi-1 was scored on a semi-quantitative scale, the percentage of positive tumor cells was recorded and divided as follows: negative (< 10% of tumor cells positive), locally positive (10%-50% of tumor cells positive), and diffusely positive (> 50% of tumor cells positive).

Statistical analysis

Data were expressed as mean ± SD and analyzed using the Stata v9.0-CYGiSO bin (Computer Resource Center, USA). The significance of differences among groups was determined by Student's *t* test and χ^2 test or Fisher's exact test. The difference in free survival between groups was analyzed by the Kaplan-Meier method and log-rank test. The starting point for calculating free survival was the date of surgery, and the endpoint was the date of death. Statistical significance was assessed at the two sided 5% level, and *P* values less than 0.05 were considered statistically significant.

RESULTS

Quantitative real-time PCR analysis

Clinical follow-up was made in 76 patients. The comparative expression levels were determined as a ratio between the Bmi1 and the housekeeping gene (β-actin) to correct for variation in the amounts of mRNA. The 5-year survival rate was 37.46%. The Bmi1 mRNA level was higher in the cancerous tissues than that in the non-cancerous tissues (2.181 ± 2.158 *vs* 0.931 ± 0.894, $P = 0.0152$). The Bmi1 expression level was unaffected by gender and age. The expression level of Bmi1 mRNA was much lower at the Ⅰ-Ⅱ stage than that at the Ⅲ-Ⅳ stage, which varied inversely with the differentiation grade, and was lower in cases without metastatic lymph nodes than in those with metastatic lymph nodes (Table 1). Based on the detection of Bmi-1 median expression level (1.085), patients were divided into the down-expression group (Bmi1 mRNA level < 1.085) and the up-expression group (Bmi1 mRNA level > 1.085), and the accumulated survival rate was higher in the former than that in the latter (Figure 3).

Protein analysis

Bmi1 oncoprotein expression was diffusely positive, focally positive and negative in 44, 16 and 10 of 70 ESCC cases, respectively. Compared with three, two and five of 10 adjacent non-cancerous cases, Bmi1 protein was significantly increased in ESCC samples ($P = 0.027$). Analysis of protein localization in ESCC cells was made, and the tumor cells with Bmi1 staining were divided

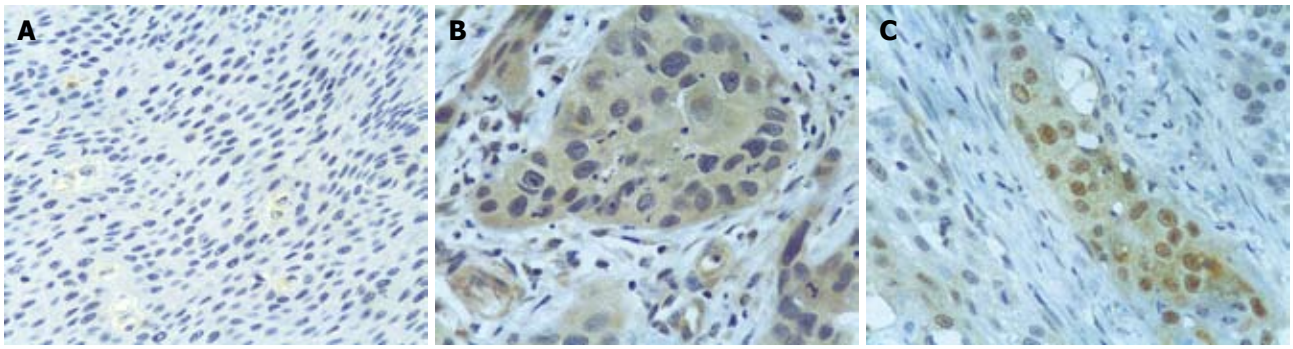

Figure 4 Immunohistochemical staining of ESCC using antibody to Bmi-1. A: Bmi1 protein expression in adjacent non-cancerous tissues (× 400); B: Cytoplasm staining of Bmi1 in ESCC cells (× 400); C: Intense Bmi-1 staining in the nucleus in ESCC cells (× 400). The brown staining under light microscopy indicates positivity.

Table 1 Associations of Bmi-1 mRNA expression in ESCC tissues with clinicopathological characteristics (mean ± SD)

Parameter	Bmi-1 mRNA	*P*
Age (yr)		
≥ 60	2.312 ± 2.171	0.3816
< 60	2.167 ± 2.045	
Gender		
Male	2.402 ± 2.359	0.2133
Female	1.770 ± 1.690	
Lymph node metastases		
Yes	3.580 ± 2.487	0.0003
No	1.703 ± 0.758	
Stage		
Ⅲ/Ⅳ	3.827 ± 2.673	0.0001
Ⅰ/Ⅱ	1.590 ± 0.735	
Histological grade		
Ⅰ	0.881 ± 0.418	0.000
Ⅱ	1.858 ± 0.979	
Ⅲ	3.580 ± 2.487	

Quantitative real-time PCR was employed to identify Bmi1 mRNA expression. The comparative expression levels were determined as a ratio between Bmi1 and housekeeping gene (β-actin) to correct variation in the amount of mRNA.

Table 2 Relationship between Bmi-1 protein expression in ESCC tissues and clinicopathological status

Parameter	> 50%	10%-50%	< 10%	*P*
Age (yr)				
≥ 60	23	9	4	0.784
< 60	21	7	6	
Histological grade				
Ⅰ	7	3	3	0.079
Ⅱ	28	5	6	
Ⅲ	9	8	1	
Lymph node metastases				
+	11	9	3	0.073
-	33	7	7	
Stage				
Ⅲ/Ⅳ	9	4	1	0.691
Ⅰ/Ⅱ	35	12	9	
Location				
Nucleus	0	7	8	0.000
Cytoplasm	30	18	16	
Both nucleus and cytoplasm	5	15	20	

ESCC was subjected to immunohistochemistry using antibodies to Bmi1. χ^2 test was used to detect the difference between Bmi1 oncoprotein and clinicopathological status.

into three categories, referring to both the nucleus and cytoplasm. In all 70 tested ESCC tissues, Bmi1 presented highly intense expression in both nucleus and cytoplasm with varied degrees, accompanied by less or even no expression in the nucleus, with significant differences (Figure 4). The positivity of the oncoprotein in samples of histological grade Ⅲ was more frequent than that of grade Ⅱ, but no significant differences were observed between other differentiated grades ($P = 0.031$). No relationship was found between the Bmi1 protein expression and lymph node metastases, pathological staging and cell differentiation (Table 2). Clinical follow-up was made in 67 patients. The 5-year survival rate was 40.01%. There was no statistical difference in survival rates between groups according to the Bmi1 expression ($P = 0.1704$).

DISCUSSION

ESCC is a major cause of morbidity and mortality worldwide[1], and it is significant to identify a biological genetic molecular marker related to its pathophysiological processes.

Epigenetic aberrations, the heritable changes in gene expression that occur in chromatin structure including DNA methylation, histone post-translational modifications and nucleosomal remodeling, rather than the DNA sequence, are involved in cancer development[17-20]. Bmi1, the first PcG protein found, is a chromatin modifier implicated in the tumorigenesis through negatively regulating the gene expression such as the INK4A locus, which is thought to regulate p53 and the Rb signaling pathway in cooperation with c-myc[5,6,19,21]. In this retrospective study, we examined the Bmi1 expression and investigated its impact on ESCC patients.

Bmi1 mRNA expression was significantly higher in the ESCC samples than in the adjacent non-cancerous tissues, and so was Bmi1 protein expression, which indicated that Bmi1 plays an important role in the development of ESCC, and has diagnostic value.

Dirks[22] has reported that Bmi1-deficient tumors may be less aggressive because they have fewer stem cells. Bmi1 expression is also found inversely correlated with the differentiation grade of clear cell carcinoma

and is involved in tumor progression[23]. Our data are in agreement with the findings by previous publications that the acquisition of metastatic ability of tumor cells is considered a late event in the evolution of malignant tumors. We found that the Bmi1 mRNA expression was higher in the stage Ⅰ/Ⅱ tissues than in stage Ⅲ/Ⅳ, significantly lower in patients without metastatic lymph nodes, and inversely related to cell differentiation. The oncoprotein was more frequently observed in tissues with poorer differentiation. These results suggest that Bmi1 expression may not be required for initiation of ESCC but is required for its progression. It may be a guide for postoperative therapy and a differentiation marker in ESCC with high malignancy. Furthermore, we discovered that the accumulated survival of patients in the up-expression group was much shorter than that of patients in the down-expression group, which may predict survival in ESCC patients.

It was interesting to note that Bmi1 protein expression was negatively correlated with malignant grade, including lymph node metastasis and advanced pathological stage. This may have been because the samples for protein analysis were obtained at different periods than those for mRNA analysis, which resulted in a different selection bias. Also, the number of lymph nodes resected by different operators varied, and the lymph nodes removed during surgery for pathological diagnosis may have been misdiagnosed as metastatic lymph nodes.

The PcG protein Bmi1 showed abundant nuclear expression in prostate cancer, colorectal cancer and gastric carcinoma[11,15,24]. However, in our study, cytoplasmic staining appeared in most of the tumor cells with less or even no expression in the nucleus alone, which suggests that Bmi1 produces a marked effect on the development of ESCC, mainly in the cytoplasm. This is inconsistent with the PcG pathway activation hypothesis that states that increased Bmi1 expression in cancer cells is associated with elevated levels of H2AubiK119 and H3metK27 histones, which suppress the expression of the INK4a/ARF locus in the nucleus[21].

The mechanisms of Bmi1 up-expression that induce adverse pathological and clinical features in ESCC patients are poorly understood. Some previous studies have shown that Bmi1 expression is a potential escape mechanism and associated with markedly increased likelihood of treatment failure and disease relapse after surgery[25,26]. Qin *et al*[27] have found that down-regulation of Bmi-1 enhances 5-fluorouracil-induced apoptosis in nasopharyngeal carcinoma cells and have suggested that the combination of 5-FU treatment and Bmi-1 depletion might be a potential clinical strategy for cancer chemotherapy.

However, we believe that further investigations on larger series of ESCC patients, including clinical follow-up and novel molecular techniques, are needed to confirm our conclusions. Whether Bmi1 can be used for accurate prediction of ESCC and its potential chemosensitivity to current pharmaceutical treatment needs further study.

COMMENTS

Background

Bmi1, a member of the polycomb group and a component of the polycomb repressive complex1, has been considered as a oncogene involved in many solid and hematological malignant tumors, and it may be of diagnostic and prognostic relevance. However, to date, no report about the role of Bmi1 in esophageal squamous cell carcinoma (ESCC) has been made. However, the up-regulation of c-Myc and the down-regulation of p53 and p16 in ESCC tissues make it plausible that Bmi1 may play an important role in the initiation and progression of ESCC.

Research frontiers

This research, for the first time, investigated the expression of Bmi1 and its clinicopathological role in patients with ESCC.

Innovations and breakthroughs

A significant upregulation of Bmi1 was observed in cancerous tissues in contrast to adjacent non-cancerous paraffin-embedded esophageal specimens at the mRNA and the protein level by quantitative real-time polymerase chain reaction (PCR) and immunohistochemistry. Furthermore, overexpression of Bmi1 positively correlated with lymph node metastases, pathological stage and differentiation grade at the mRNA level.

Applications

Bmi1 may act as a guide for the postoperative therapy and a differentiation marker of ESCC with high malignancy, and for prediction of the survival of ESCC patients.

Peer review

The authors investigated the expression of Bmi1 at the mRNA and protein level in patients with ESCC in comparison with healthy adjacent tissue, by real-time PCR and immunohistochemistry. They observed a significant up-regulation of Bmi1 in tumor tissues in contrast to healthy control tissues. Up-regulation correlated positively with lymph node metastases, stage and histological grading at the mRNA level. However, the histological analysis showed no such correlation. The authors conclude that Bmi1 expression is common in ESCC and may serve as a marker to predict lymph node metastasis and survival in ESCC patients.

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Polymorphisms of alcohol dehydrogenase-2 and aldehyde dehydrogenase-2 and esophageal cancer risk in Southeast Chinese males

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Abstract

AIM: To evaluate the impact of alcohol dehydrogenase-2 (ADH2) and aldehyde dehydrogenase-2 (ALDH2) polymorphisms on esophageal cancer susceptibility in Southeast Chinese males.

METHODS: Two hundred and twenty-one esophageal cancer patients and 191 healthy controls from Taixing city in Jiangsu Province were enrolled in this study. ADH2 and ALDH2 genotypes were examined by polymerase chain reaction and denaturing high-performance liquid chromatography. Unconditional logistic regression was used to calculate the odds ratios (OR) and 95% confidence interval (CI).

RESULTS: The ADH G allele carriers were more susceptible to esophageal cancer, but no association was found between ADH2 genotypes and risk of esophageal cancer when disregarding alcohol drinking status. Regardless of ADH2 genotype, ALDH2G/A or A/A carriers had significantly increased risk of developing esophageal cancer, with homozygous individuals showing higher esophageal cancer risk than those who were heterozygous. A significant interaction between ALDH2 and drinking was detected regarding esophageal cancer risk; the OR was 3.05 (95% CI: 1.49-6.25). Compared with non-drinkers carrying both ALDH2 G/G and ADH2 A/A, drinkers carrying both ALDH2 A allele and ADH2 G allele showed a significantly higher risk of developing esophageal cancer (OR = 8.36, 95% CI: 2.98-23.46).

CONCLUSION: Both ADH2 G allele and ALDH2 A allele significantly increase the risk of esophageal cancer development in Southeast Chinese males. ALDH2 A allele significantly increases the risk of esophageal cancer development especially in alcohol drinkers. Alcohol drinkers carrying both ADH2 G allele and ALDH2 A allele have a higher risk of developing esophageal cancer.

Key words: Alcohol dehydrogenase-2; Aldehyde dehydrogenase-2; Gene polymorphisms; Alcohol drinking; Esophageal cancer

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INTRODUCTION

There is epidemiological evidence showing that alcohol intake is associated with increased esophageal cancer risk[1]. Acetaldehyde, the oxidative metabolite of ethanol, is recognized to be carcinogenic in animals and suspected to have similar effects in humans[2]. Ethanol is oxidized to acetaldehyde and then to acetate by alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH), both of which have genetic

polymorphisms. People homozygous for the ALDH2*2 allele (Glu487Lys, Lys or A allele) do not have any ALDH activity. Heterozygous individuals carrying the reference (G) and variant (A) alleles (ALDH2*1/2, or A/G) show only 1/16 of the activity seen in ALDH2*1 homozygotes (G/G)[3-5]. ADH2*2 allele (Arg47His, His or A allele) encodes a superactive subunit of ADH2 and that superactive ADH2*2 homodimer has about a 40 times higher Vmax than the less-active ADH2*1/2*1 form of ADH2[3,4]. Therefore, shortly after alcohol drinking, individuals carrying both variant ADH2 and ALDH2 would accumulate a large amount of aldehyde that cannot be efficiently oxidized to the non-toxic acetic acid. Different combinations of ADH2 and ALDH2 genotypes may influence the individual susceptibility to cancer.

Taixing city, located in the middle part of Jiangsu Province, China, has relatively high incidence and mortality rates for esophageal cancer (in 2005, the age-adjusted mortality rate was 53.66 per 100 000 for esophageal cancer). Our previous study has shown that more than 40% of adult residents in Taixing drink wine and that drinking is a risk factor for esophageal cancer in this area[6]. We have also shown relationships between ALDH2 and the risk of esophageal cancer, but no statistically significant association was found[7]. In this study, we increased the sample size to define the individual and combined roles of ADH2, ALDH2 polymorphisms and drinking habits in the risk analysis for esophageal cancer development in Southeast Chinese males.

MATERIALS AND METHODS

Study subjects

We recruited male patients who were histopathologically diagnosed as having esophageal carcinoma from January 2005 to December 2006. Population-based male controls were recruited from healthy residents in the villages or towns where cases resided. All study subjects have completed a questionnaire administrated by a trained interviewer, covering residential, occupational, social, living style, psychological and economical factors. The interviewer then collected blood samples of subjects from a peripheral vein after obtaining their oral informed consents. The collected blood samples were shipped to the public health center within a day. Buffy coat was then separated and stored at -30℃. We defined a drinker as a person who drinks at least once per week (alcohol intake more than 40 g) and continuously drinks for at least half the year. A few patients and residents refused to participate in our study, but the overall response rate was 97% for patients and 95% for controls, respectively. The Ethics Committee of Jiangsu Provincial Institute of Cancer Research approved this study. Associations could not be assessed in women because of sparse drinking habits.

DNA extraction and genotyping of ADH2 and ALDH2

Whole blood was collected into EDTA-coated tubes and centrifuged for 15 min. The buffy coat layer was isolated. Genomic DNA was extracted from 200 μL of buffy coat using a Qiagen QIAamp DNA blood mini kit (QIAGEN Inc., Valencia, CA). Genotyping of ADH2 and ALDH2 was determined by polymerase chain reaction (PCR) and denaturing high-performance liquid chromatography (DHPLC).

The sequences of primers used in this study are F: 5'-GGGCTTTAGACTGAATAACCTTGG-3' and R: 5'-AGGGAAAGAGGAAACTCCTGAA-3' for ADH2 Arg47His, and F: 5'-TGCTATGATGTGTTTGGAGCC-3' and R: 5'-GGCTCCGAGCCACCA-3' for ALDH2 Glu487Lys. Reactions were carried out in a total volume of 25 μL containing 20 pmol of each primer, 0.25 mmol/L each dNTPs, 2.0 mmol/L $MgCl_2$, 2.5 μL 10 × buffer, 1 IU hotTag polymerase and 0.5 μL genomic DNA. PCR conditions were as follows: denaturation at 95℃ for 7 min, followed by 35 cycles at 95℃ for 30 s, at 62℃ for 30 s, at 72℃ for 30 s, and a final extension at 72℃ for 5 min. The products were denatured at 94℃ for 4 min, and their temperature was declined to 25℃ step by step according to 0.1℃/s.

Transgenomic WAVE DNA fragment analysis system (WAVE-300, Transgenomic, USA) and associated WAVEMAKER software were used for genotyping. An aliquot (5 μL) of the PCR products was directly injected into a DNASep column. The column mobile phase for sample elution consisted of a mixture of buffer A [0.1 mol/L triethylammonium acetate (TEAA)] and buffer B (0.1 mol/L TEAA with 25% acetonitrile). Samples were eluted at a linear gradient of buffer B over a 4.5-min period at a constant flow rate of 0.9 mL/min. For each DNA region, DHPLC conditions were established by a titration analysis at 1-3℃ above and below the mean melting temperature predicted by software simulation. There were three genotypes: namely G/G, G/A, and A/A, for ADH2 Arg47His and ALDH2 Glu487Lys, respectively.

Statistical analysis

All analyses were done with the SAS (version 6.02) and Epi-info (version 6.04) statistical package. Odds ratios (OR) and 95% confidence intervals (CI) were adjusted by unconditional logistic regression analysis. Gene-environment interactions were evaluated by additive model and expressed in terms of synergy index (S) and attributable proportions of interaction (API)[8]. The Mantel-Haenszel χ^2 method was used to test for significant associations between the ADH2 or ALDH2 genotype and cancer risk.

RESULTS

Four hundred and twelve Jiangsu males were enrolled in this study. Numbers of subjects were 221 cases with esophageal cancer and 191 controls (Table 1). The proportional distributions of age, occupation, education, smoking and drinking did not significantly differ between cases and controls, but the proportional distributions

Table 1 Background characteristics of cases and their controls

	Controls *n* (%)	Cases *n* (%)	χ^2 MH	*P*
Age (yr)				
<50	10 (4.53)	8 (4.19)		
50-59	57(25.79)	60 (31.41)		
60-69	98 (44.34)	80 (41.88)		
> 70	56 (25.34)	43 (22.52)	0.91	0.341
Total	221	191		
Income (yuan/year per person)				
Ten years before	2097	3040		< 0.01
Recent years	3629	4746		< 0.01
Drinking status				
Non-drinker	96 (43.44)	94 (49.21)		
Drinker	125 (56.56)	97 (50.79)	1.38	0.24
Smoking status				
Non-smoker	70 (31.67)	58 (30.37)		
Smoker	151 (68.33)	133 (69.63)	0.78	0.08

Table 2 ORs and their 95% CIs for esophageal cancer with reference to ALDH2 and ADH2 polymorphisms

	Controls *n* (%)	Cases *n* (%)	OR[1] (CI)	OR[2] (CI)
ALDH2 genotype				
G/G	90 (40.73)	114 (59.69)	1.00	1.00
G/A	89 (40.27)	66 (34.55)	1.71 (1.10-2.66)	1.70 (1.08-2.68)
A/A	42 (19.00)	11 (5.96)	4.84 (2.25-10.61)	5.69 (2.51-12.18)
G/A+A/A	131 (59.27)	77 (40.51)	2.15 (1.43-3.26)	2.19 (1.43-3.34)
ADH2 genotype				
A/A	106 (47.96)	108 (56.54)	1.00	1.00
A/G	96 (43.44)	75 (39.27)	1.30 (0.85-1.99)	1.21 (0.79-1.86)
G/G	19 (8.60)	8 (4.19)	2.42 (1.02-5.77)	2.78 (1.06-7.29)
A/G+G/G	115 (52.04)	83 (43.46)	1.41 (0.96-2.08)	1.34 (0.89-2.04)
Allele frequencies				
ALDH2				
G	269 (60.86)	294 (76.96)	1.00	
A	173 (39.14)	88 (23.04)	2.15 (1.57-2.95)	
ADH2				
A	308 (69.68)	291 (76.18)	1.00	
G	134 (30.32)	91 (23.82)	1.39 (1.01-1.92)	

[1]Crude OR; [2]Adjusted odds ratios (ORs) were adjusted for income.

of income (ten years before and recent years) were significant lower in cases than in controls (4.52 and 3.64 for *T* value, $P < 0.01$).

As shown in Table 2, the frequency of ALDH2 G/G, G/A and A/A genotypes were 40.73%, 40.27% and 19.00% in cases and 59.69%, 34.55% and 5.96% in controls respectively. The distribution of the ALDH2 genotypes was significant different between controls and cases ($\chi^2 = 22.30$, $P < 0.01$). The frequency of ADH2 A/A, A/G and G/G genotypes demonstrated no significant differences between cases and controls ($\chi^2 = 4.92$, $P = 0.085$). The allelic distribution of ADH2 and ALDH2 polymorphisms was in Hardy-Weinberg equilibrium ($P > 0.05$).

As for income-adjusted odds ratio, compared with ALDH2 G/G carriers, the OR was 1.70 (95% CI: 1.08-2.68) for G/A carriers, 5.69 (95% CI: 2.51-12.18) for the A/A carriers and 2.19 (95% CI: 1.43-3.34) for the two genotypes combined. Compared with the subjects with ADH2 A/A genotype, subjects with G/G genotypes had an increased OR of 2.78 (95% CI: 1.06-7.29). As for allelic comparison, the OR was 2.15 (95% CI: 1.57-2.95) for ALDH2 A allele and 1.39 (95% CI: 1.01-1.92) for ADH2 G allele carriers.

Table 3 Interaction between ALDH2 and ADH2 genotype and the ORs for esophageal cancer

ADH2	ALDH2	Cases	Controls	OR[1] (95% CI)	OR[2] (95% CI)
A/A	G/G	44	68	1.00	1.00
G/A+G/G	G/G	46	46	1.55 (0.89-2.70)	1.46 (0.79-2.70)
A/A	G/A	40	33	1.87 (1.03-3.40)	1.93 (0.99-3.75)
G/A+G/G	G/A	49	33	2.29 (1.28-4.11)	2.10 (1.13-3.91)
A/A	A/A	22	7	4.98 (1.91-12.33)	5.28 (1.88-14.83)
G/A+G/G	A/A	20	4	7.73 (2.48-24.13)	12.22 (2.62-56.91)

[1]Crude OR; [2]ORs were adjusted for income.

Regardless of ADH2 genotype, ALDH2G/A or A/A carriers were found to have significantly increased risk of developing esophageal cancer. ALDH2 A/A homozygotes have higher esophageal cancer risk than ALDH2G/A heterozygotes. As compared to the subjects with ADH2A/A and ALDH2 G/G genotypes (double wild type), those with variant alleles for both ADH2 (G allele) and ALDH2 (A allele) had a significantly increased OR. ALDH2 A/A homozygotes who were also carrying ADH2 G allele had the highest OR of 12.22 (95% CI: 2.62-56.91) (Table 3).

The ALDH2 A/A genotype alone showed a moderate increase of esophageal cancer risk in both drinkers and non-drinkers (Table 4). No significant relationship was found in analysis of ADH2 genotypes. Compared with non-drinkers with both ALDH2 G/G and ADH2 A/A genotypes, drinkers with ALDH2 A and ADH2 G alleles showed a significantly elevated risk of esophageal cancer (OR = 8.36, 95% CI: 2.98-23.46)

The OR for esophageal cancer among alcohol drinkers with ALDH2 A allele was markedly increased to 3.05 (95% CI: 1.49-6.25) compared to non-drinkers with ALDH2 G/G genotypes (Table 5). A significant gene-environment interaction between alcohol drinking and ALDH2 was observed for esophageal cancer risk (S = 2.93). The population attributable risk due to alcohol drinking by ALDH2 A allele carriers was estimated to be 41% for esophageal cancer (API = 0.41).

DISCUSSION

Our previous studies showed that drinking was associated with increased esophageal, stomach and liver cancer in Taixing[6]. We also found that it was not ADH2 but ALDH2 polymorphisms that had a significant interaction with heavy alcohol consumption in the development of hepatocellular carcinoma (HCC)[9]. In the present study, both ADH2 G allele and ALDH2 A allele significantly increased the risk of esophageal cancer development. ALDH2 A allele significantly increases the risk of esophageal cancer development

Table 4 Analysis of ALDH2 and ADH2 genotypes and risk of esophageal cancer with reference to drinking habits

Genotypes		Non-drinker			Drinker		
		Cases	Controls	OR[1] (95% CI)	Cases	Controls	OR[1] (95% CI)
ALDH2							
G/G		26	42	1.00	64	72	1.00
G/A		43	44	1.29 (0.65-2.55)	46	22	2.47 (1.27-4.82)
A/A		27	8	4.67 (1.63-13.38)	15	3	8.63 (2.07-35.95)
G/A+A/A		70	52	1.78 (0.94-3.37)	61	25	3.08 (1.65-5.78)
ADH2							
A/A		50	53	1.00	56	55	1.00
G/A		42	38	1.31 (0.70-2.46)	54	37	1.18 (0.64-2.16)
G/G		4	3	2.10 (0.35-12.54)	15	5	2.90 (0.85-9.90)
G/A+G/G		46	41	1.37 (0.74-2.54)	69	42	1.36 (0.76-2.43)
ALDH2	ADH2						
G/G	A/A	10	28	1.00	34	40	2.02 (0.79-5.17)
G/A+A/A	G/A+G/G	30	27	2.84 (1.10-7.31)	39	10	8.36 (2.98-23.46)

[1]ORs were adjusted for income.

Table 5 Interaction between alcohol drinking and ALDH2 genotype and the ORs for esophageal cancer

Genotype[1]	Drinker[2]	Cases	Controls	OR[1] (95% CI)
-	-	26	42	1.00
-	+	64	72	0.92 (0.48-1.78)
+	-	70	52	1.78 (0.94-3.37)
+	+	61	25	3.05 (1.49-6.25)

[1]-: ALDH2G/G; +: ALDH2G/A and A/A; ORs were adjusted for income; [2]-: Non-drinker; +: Drinker.

especially in alcohol drinkers. Alcohol drinkers carrying both ADH2 G allele and ALDH2 A allele have a higher risk of developing esophageal cancer.

There is no doubt that the differences in environment exposures/lifestyle influence the genetic susceptibility to cancer. There have been a lot of papers regarding the relationship between ADH2 and ALDH2 polymorphisms and esophageal cancer susceptibility. Chao *et al*[10] found that Chinese alcoholic patients with the ADH G and ALDH2 A allele were more susceptible to esophageal cancer. Many studies found that the inactive ALDH2 genotypes had a significantly increased risk for developing esophageal cancer and that a gene-environment interaction exists between alcohol drinking and the inactive ALDH2 genotypes[2,11-16]. Boonyaphiphat *et al*[17] did not find ALDH2 increased the risk significantly (OR of ALDH G/A 1.57, 95% CI: 0.89-2.76). However, the combined at risk genotypes, ADH A/A and ALDH G/A increased risk by four-fold and heavy drinkers > 60 g/d harboring ADH A/A or ALDH G/A had about an 11-fold increased risk. Our previous study showed no statistically significant association between ALDH2 and esophageal cancer susceptibility[7]. However, in this study with a larger sample size, we found that the ALDH2 A allele showed a moderately increased risk for esophageal cancer as compared with ALDH2 G/G carriers, and significant gene-environment interactions between alcohol drinking and ALDH2 were observed regarding esophageal cancer risk (S = 2.93). The population attributable risk due to alcohol drinking by ALDH2 A allele carriers was estimated to be 41% for esophageal cancer. Yokoyama *et al*[18] also found that an extraordinarily high proportion of excessive risk for esophageal cancer in Japanese males can be attributed to drinking by persons with inactive heterozygous ALDH2 (68.5%). Aldehyde dehydrogenase-2 generates acetic acid from acetaldehyde metabolism and its activity correlates with *in vivo* acetaldehyde concentration. Thus, diminished ALDH2 enzyme activity and consequent higher concentrations of acetaldehyde can be risk factors for esophageal cancer. In this study, we, for the first time, report that ALDH2 A/A homozygotes have higher esophageal cancer risk than ALDH2 G/A homozygotes, which is consistent with the different ALDH2 enzyme activity resulting from A/A and G/G genotypes. Literature has shown that after drinking, the blood acetaldehyde concentrations in those with ALDH2 A/A and G/A were 19- and 6-fold higher than in those with G/G genotype, respectively[19].

In this study, we found the ADH G allele carriers were more susceptible to esophageal cancer, but no association was found between ADH2 genotypes and risk of esophageal cancer when disregarding drinking status. Compared with non-drinkers carrying both ALDH2 G/G and ADH2 A/A, drinkers carrying both ALDH2 A allele and ADH2 G allele showed a significantly higher risk of developing esophageal cancer (OR = 8.36, 95% CI: 2.98-23.46). The inactive ADH2 genotype has also been demonstrated to enhance the risk of esophageal cancer among alcoholics and the general population. The inactive ALDH2 genotype and ADH2 genotype carriers have higher risk of developing esophageal cancer, especially among alcohol drinkers[11,13-18]. These findings conflicts with those demonstrating that the enzyme activity in ADH G allele was much higher than that of A allele. Yoshihara *et al*[20] showed that there were no significant differences in blood ethanol and acetaldehyde concentrations between volunteers with ADH2*1 and without ADH2*1. Thus, the mechanism of the ADH2 polymorphism involved

in esophageal cancer risk may be associated with, not acetaldehyde, but a direct involvement of ethanol.

In summary, this study found that polymorphisms of the ADH2 and ALDH2 genes were significantly associated with the risk of esophageal cancer in Southeast Chinese males. Significant gene-environment interactions between alcohol drinking and ALDH2 were observed in esophageal cancer risk. Significant interactions between ADH2 and ALDH2 polymorphisms were also observed. These findings can provide additional information about the role of alcohol in esophageal cancer risk in Chinese populations. For individuals with ALDH2 A/A or G/A genotypes, reducing alcohol consumption may help lower their risk for esophageal cancer.

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COMMENTS

Background

Esophageal cancer is the most common cancer in China. There is epidemiological evidence that alcohol intake is associated with an increased esophageal cancer risk. Alcohol dehydrogenase-2 (ADH2) and aldehyde dehydrogenase-2 (ALDH2) have a strong impact on alcohol metabolism. The authors' previous study has shown that more than 40% of adult residents in Taixing drink wine and that drinking is a risk factor for esophageal cancer in this area. However, no statistically significant association between ALDH2 and the risk of esophageal cancer was found. In this study, the authors increased the sample size to define the individual and combined roles of ADH2, ALDH2 polymorphisms and drinking habits in the risk for esophageal cancer development in Chinese males.

Innovations and breakthroughs

The present study showed that polymorphisms of the ALDH2 genes were significantly associated with the risk of esophageal cancer in Southeast Chinese males. Significant gene-environment interactions between alcohol drinking and ALDH2 were observed in esophageal cancer risk. Significant interactions between ADH2 and ALDH2 polymorphisms were also observed.

Applications

This research showed the genetic risk factors and the role of gene-environment interactions in identifying individuals at risk of esophageal cancer, which have certain theoretical and application values for studying the etiology of esophageal cancer and its prevention.

Terminology

ADH2: A zinc-containing enzyme which oxidizes primary and secondary alcohols or hemiacetals in the presence of NAD. In alcoholic fermentation, it catalyzes the final step of reducing aldehyde to alcohol in the presence of NADH and hydrogen. ALDH2: An enzyme that oxidizes aldehyde in the presence of NAD+ and water to acid and NADH. Genetic polymorphisms: The regular and simultaneous occurrence of two or more discontinuous genotypes in a single interbreeding population. The concept includes differences in genotypes ranging in size from a single nucleotide site to large nucleotide sequences visible at a chromosomal level.

Peer review

This study provides more information on the ADH2 and ALDH2 polymorphisms of esophageal cancer in Southeast Chinese males and the findings support the previous results that the risk of esophageal cancer increases in subjects carrying ADH2*1 allele (G allele) and ALDH2*2 allele (A allele) in an overall population, and especially ALDH2*2 allele (A allele) in alcohol drinkers. These epidemiological findings might help construct a prevention strategy against esophageal cancer.

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Diagnostic effect of capsule endoscopy in 31 cases of subacute small bowel obstruction

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Abstract

AIM: To evaluate the effectiveness and safety of capsule endoscopy (CE) in patients with recurrent subacute small bowel obstruction.

METHODS: The study was a retrospective analysis of 31 patients referred to hospital from January 2003 to August 2008 for the investigation of subacute small bowel obstruction, who underwent CE. The patients were aged 9-81 years, and all of them had undergone gastroscopy and colonoscopy previously. Some of them received abdominal computed tomography or small bowel follow-through.

RESULTS: CE made a definitive diagnosis in 12 (38.7%) of 31 cases: four Crohn's disease (CD), two carcinomas, one intestinal tuberculosis, one ischemic enteritis, one abdominal cocoon, one duplication of the intestine, one diverticulum and one ileal polypoid tumor. Capsule retention occurred in three (9.7%) of 31 patients, and was caused by CD (2) or tumor (1). Two with retained capsules were retrieved at surgery, and the other one of the capsules was spontaneously passed the stricture by medical treatment in 6 mo. No case had an acute small bowel obstruction caused by performance of CE.

CONCLUSION: CE provided safe and effective visualization to identify the etiology of a subacute small bowel obstruction, especially in patients with suspected intestinal tumors or CD, which are not identified by routine examinations.

Key words: Capsule endoscopy; Small bowel obstruction; Capsule retention

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INTRODUCTION

Small bowel obstruction is a frequent cause of acute abdomen. The definitive diagnostic rate is not high using traditional radiographic evaluation, such as plain film radiography, abdominal computed tomography (CT), or small bowel follow-through. Some reports have demonstrated that capsule endoscopy (CE) is superior to radiographic examination and push enteroscopy in the investigation of intestinal diseases, especially for obscure gastrointestinal bleeding or suspected Crohn's disease (CD)[1-4]. Although capsule retention is a relatively infrequent complication, small bowel obstruction and strictures have been considered contraindications to CE. It is interesting to note that there is a controversy about this contraindication in the literature. The goal of the present study was to evaluate the safety and effectiveness of CE in patients with small bowel obstruction.

MATERIALS AND METHODS

Subjects

Between January 2003 and August 2008, 31 patients underwent CE for the investigation of small bowel obstruction, who had previously received gastroscopy and colonoscopy, abdominal CT or small bowel follow-

Table 1 Clinical findings and outcomes of CE or surgery

Patient	Gender/age (yr)	Surgical history/ NSAID use	Prior examinations	GI transit time (min)	CE or surgical findings	Follow-up (mo)
1	M/43	None	EGD, colonoscopy (-), AXR	319	Abdominal cocoon	17
2	F/18	Appendectomy	EGD, colonoscopy (+), AXR	387	CD	53
3	M/74	None	EGD, colonoscopy (-), AXR	329	Normal	54
4	F/69	None	EGD, colonoscopy, SBFT (-), AXR	295	Normal	53
5	M/54	None	Colonoscopy, SBFT (±), AXR	CE retention	CD	29
6	M/9	Intussusception	EGD, colonoscopy (-), AXR	205	Normal	16
7	F/67	None	EGD, colonoscopy (-), AXR	Not pass	Ischemic enteritis	Lost in 1[1]
8	F/36	None	EGD, colonoscopy, CTE (-), AXR	308	Normal	27
9	M/46	None	CTE (±), SBFT (±), AXR	461	Tumor	15
10	F/37	Abdominal delivery	EGD, colonoscopy, SBFT (-), AXR	247	Normal	17
11	F/52	None	EGD, colonoscopy, CTE, SBFT (-)	CE retention	Tumor	2
12	F/52	None	EGD, colonoscopy, CTE, SBFT (-)	Not pass	Normal	1
13	F/62	None	EGD, colonoscopy, CTE, SBFT (-)	Not pass	Normal	2
14	M/57	None	EGD, CTE, SBFT (-), AXR	324	Normal	33
15	M/31	None	EGD, colonoscopy (-), AXR	250	Normal	32
16	F/32	None	EGD, colonoscopy (±), CTE (-)	446	Normal	30
17	M/53	None	CTE (+), EGD/colonoscopy (-),	346	Normal	33
18	F/31	Abdominal delivery	EGD/colonoscopy (-), US/CTE (+)	425	TB	30
19	M/22	None	EGD/colonoscopy, SBFT (-), AXR	340	Normal	3
20	M/46	Small bowel resection	EGD/colonoscopy, CTE (-), AXR	296	Normal	3
21	M/81	None	Colonoscopy (-), AXR	378	Normal	Lost[2]
22	F/54	Tubal ligation	EGD/colonoscopy, CTE (-)	458	Normal	41
23	M/75	None	EGD/colonoscopy (-), AXR	327	Normal	Death in 24
24	M/53	None	EGD/colonoscopy (-), AXR	421	CD	51
25	M/60	None	EGD/colonoscopy, SBFT (-)	465	Intestinal diverticulum	5
26	F/52	None	EGD/colonoscopy, CTE (±)	465	Normal	16
27	M/32	None	EGD/colonoscopy (-), AXR	293	Normal	39
28	F/54	Tubal ligation	EGD/colonoscopy, SBFT (-)	354	Normal	36
29	F/59	None	EGD/colonoscopy, MRI (-), AXR	349	Ileal polypoid tumor	Lost[2]
30	M/9	None	EGD/colonoscopy, CTE (-), AXR	Not pass	Duplication of intestine	12
31	F/65	None	EGD/colonoscopy, SBFT (-), AXR	CE retention	CD	14

EGD: Esophagogastroduodenoscopy; AXR: Abdominal X-ray; MRI: Magnetic resonance imaging; CE: Capsule endoscopy; CTE: CT enterography; SBFT: Small bowel follow-through; US: Ultrasound; CD: Crohn's disease. (±): Suspected positive; (+): Positive; (-): Negative; Not pass: The capsule did not pass the ileocecal valve within the duration of the examination, but was not retained. GI: Gastrointestinal. [1]The patient was lost to follow-up 1 mo after surgery. [2]The follow-up was missed after CE examination.

through more than once. All previous radiological and endoscopic examinations could not identify clear etiology.

Materials

CE (Given M2A, Giving Imaging Ltd, Yoqneam, Israel) measuring 11 mm × 26 mm, which magnify images eight times, has a battery life of 6-8 h. It is used in conjunction with an imaging system including a data recorder and interpretative workstation. Continuous video-images are transmitted at a rate of two frames per second.

Methods

A total of 1121 patients underwent CE between January 2003 and August 2008. Most of them underwent CE for the evaluation of obscure bleeding or suspected CD. We identified 31 patients presenting with symptoms consistent with small bowel obstruction, and abdominal X-ray showed incomplete intestinal obstruction. All the 31 patients who were aware of an increased risk for capsule retention and the possibility for surgery received CE examination, when the symptoms of intestinal obstruction were relieved by conservative management. All the patients gave written informed consent. The medical data were retrospectively analyzed, including age, sex, medical and surgical history, follow-up, and radiographic, routine endoscopic and CE examinations.

RESULTS

The mean age of these 31 patients was 47.12 ± 18.38 years (range 9-81 years); 18 of the subjects were male and 13 were female. Seventeen of them were out-patients, 14 were in-patients, and nine had surgical histories before capsule examinations were performed. All of them had undergone gastroscopy and colonoscopy previously, but the results were negative. Twenty-three of them had undergone CT enterography or small bowel follow-through, and positive or suspected results were found in six cases. Four of the six patients achieved definitive diagnoses by CE examination, surgical or pathological biopsy, and the remaining two were false-positive.

The average gastric emptying time was 43.8 ± 36.1 min (range 4-131 min). In 15 of the 31 patients, the capsule passed the ileocecal valve within the duration of the examination. The mean small bowel transit time (based on 24 patients) was 332.2 ± 86.7 min (range 167-484 min, Table 1). In 28 of the 31 patients, the capsule was evacuated in 3 d. Capsule retention occurred in three

Table 2 Abnormalities detected on CE in patients with small bowel obstruction

Detected abnormalities (12)	Gender/age (yr)	CE retention (time)	Therapy	Post-CE obstructive symptom
CD (4)				
	F/18	No	Medical therapy	None
	M/54	Yes (1 wk)	Surgical resection	None
	M/53	No	Medical therapy	None
	F/65	Yes (6 mo)	Medical therapy	None
Tumor (2)				
Ileal neuroendocrine carcinoma	M/46	No	Surgical resection	None
Jejunal adenocarcinoma	F/52	Yes (2 wk)	Surgical resection	None
Intestinal tuberculosis (1)	F/31	No	Medical therapy	None
Ischemic enteritis (1)	F/67	No	Surgical resection	None
Abdominal cocoon (1)	M/43	No	Surgical resection	None
Intestinal diverticulum (1)	M/60	No	Medical therapy	None
Ileal polypoid tumor (1)	F/59	No	Lost to follow-up	None
Duplication of intestine (1)	M/9	No	Surgical resection	None

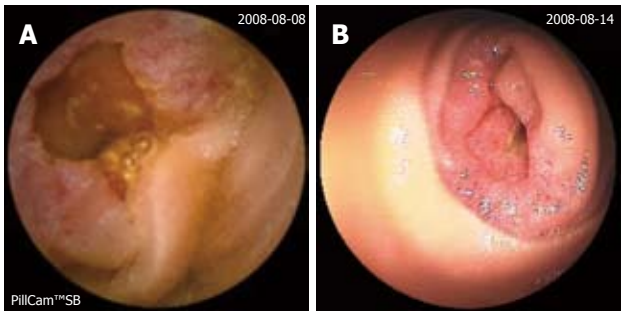

Figure 1 CE and double air-balloon endoscopic images of stenosis. A: CE shows an annuliform mass in the intestine; B: Double air-balloon endoscopy also shows an annuliform mass, but we failed to retrieve the retained capsule.

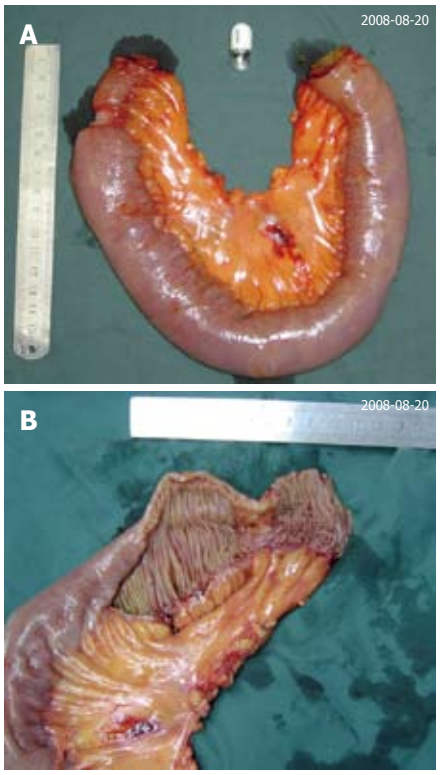

Figure 2 Surgical images of stenosis. A: A retained capsule was removed at surgery; B: An obvious stenosis was caused by jejunal adenocarcinoma.

(9.7%) cases, caused by CD or tumor, of which, were retrieved at surgery, and the other one of the capsules was spontaneously passed the stricture by medical treatment in 6 mo. None of the cases showed any symptoms of acute or subacute obstruction during CE examination.

CE disclosed definitive intestinal disease in 12 (38.7%) of the 31 patients, including four CD, two carcinoma, one intestinal tuberculosis, one ischemic enteritis, one abdominal cocoon, one intestinal duplication, one small-intestinal diverticulum and one ileal polypoid tumor (Table 2). Single or multiple ulcers were found in six patients. In three of the six, CD was diagnosed by CE images and clinical manifestations, and obvious symptom relief was achieved through treatment with mesalazine. In one of the six patients, capsule was retrieved at surgery which had not passed the stricture for 7 d, and the replacement showed CD. In another of the six patients, multiple ulcers were found with CE and double-balloon enteroscopy. CD was firstly considered according to the endoscopic findings and clinical data, but medical treatment with mesalazine did not relieve the patient's symptoms. The later BUS and CT scans showed multiple retroperitoneal lymph node enlargement, meanwhile, the purified protein derivative test was found to be positive. Pathological analysis of biopsy specimens obtained from these lymph nodes indicated tuberculosis. The patient's symptoms were relieved significantly by anti-tuberculosis treatment, therefore, intestinal tuberculosis was diagnosed. The remainder of the six was demonstrated abdominal cocoon at surgery. In one elderly patient, intestinal mucosal erosion and bleeding were found at CE examination. Later, exploratory laparotomy was performed for advanced identification of etiology and therapy, which indicated superior mesenteric artery embolus. In another case, the capsule images presented abnormal intestinal motility and CT scan showed mural thickening of the distal ileum, and finally, ileal neuroendocrine carcinoma was diagnosed by surgery. In a pediatric case, CE also showed abnormal intestinal motility. The child was treated surgically because of failure of medical treatment, which indicated duplication of the intestine. The CE findings in the remaining two cases disclosed diverticulum of the small intestine and ileal polypoid tumor. In a female patient

whose CA199 increased clearly CT, BUS scan or air-barium double contract examination were negative, but CE and double air-balloon endoscopy showed an annuliform mass, which was demonstrated to be jejunal adenocarcinoma at later surgery (Figures 1 and 2).

None of the patients had other risk factors for stricture formation, such as long-term administration of non-steroidal anti-inflammatory drug (NSAIDs) and abdominal radiotherapy. The capsule images were normal in 19 of the 31 cases. Follow-up was missed in three of the 19 cases. An elderly patient in the remaining 16 died of pulmonary infection. Small bowel obstruction did not reappear in the other 15 cases during medical treatment in the follow-up period. However, adhesive ileus could not be excluded in four of the 14 patients who had a history of abdominal surgery. The capsule findings allowed a definitive diagnosis in 12 of the 31 cases: six patients accepted surgical treatment (one CD, two tumors, one ischemic enteritis, one abdominal cocoon, one duplication). Five patients (three CD, one intestinal tuberculosis, one intestinal diverticulum) were treated medically without surgery, and no recurrence of small bowel obstruction was found in these patients during follow-up.

DISCUSSION

CE is a novel diagnostic technique that has been used increasingly for analysis of many disorders of the small intestine, such as occult gastrointestinal bleeding, suspected CD, chronic diarrhea, and protein-losing enteropathy. Although small bowel obstruction has been considered a contraindication to CE, in our series, CE was documented to be very valuable and safe in identifying the etiology of small bowel obstruction, and it was also found to be easy to swallow, painless and well tolerated by these selected patients. CE findings allowed a definitive diagnosis in 12 (38.7%) out of the 31 cases, in which CD (4/31) was the major disease inducing stricture of the intestine. This cause was consistent with that in the study of Chiefetz and Lewis[5]. In contrast, some authors have reported that NSAID-induced stricture was the major cause of capsule retention[6,7]. Recently, Mason *et al*[8] have reported that intestinal mass or radiation enteritis are the main causes of subacute small bowel obstruction. The different causes of stricture may be associated with the indications of the patients.

The incidence of retention is closely related to the selected population. The incidence of retention varies from 0%-21% in the literature as a result of the different populations and indications for examination. The highest published rate (21%) was reported in the study of Chiefetz and Lewis[5], in which CD (2/19 cases) was also noted to be the major cause of retention. In another study with a total of 102 cases[9], the rate of retention was 13% (5/38) in patients with known CD, but only 1/64 cases with suspected CD had a retained capsule. The rate of capsule retention was very low in most studies, especially when the patients were selected without suspected small bowel obstruction or intestinal stricture. In the report of Barkin and Friedman[10], the incidence was 0.75% in a large study of 900 patients who had previously normal small intestines. Most recently, Li *et al*[7] have reported 14 cases of CE retention (1.4%) in 1000 capsule examinations. It was shown that tumors or NSAID strictures were the major etiology of retention in both of these studies. In our highly selected population, capsule retention occurred in 3 of 31 cases (two CD, one tumor).

Recently, dissolving patency capsules have been used in some studies to evaluate intestinal patency in patients with small bowel strictures, before video-CE (VCE). The patency capsule[11] is composed of lactose, remains intact in the gastrointestinal tract for 40-100 h post-ingestion, and disintegrates thereafter. Spada *et al*[12] have reported that 94% (30/34) of cases with small bowel stricture passed the intact or disintegrated capsule in the stools. Expulsion was confirmed in three cases by fluoroscopy, and the remaining patient withdrew consent to the study. In addition, VCE passed uneventfully through the small bowel stricture of all 10 patients who underwent VCE following patency capsule examination. The study of Spada *et al* has suggested that the patency capsule is a safe and effective tool for evaluation of functional patency of the small bowel, even when stricture has been indicated by traditional radiology. In another multicenter study[13], in all the 106 patients with strictures, no acute ileus was induced by Agile patency capsule. However, in the study of Bovin *et al*[14], in one of the 22 cases with suspected obstructive intestinal disease and/or radiological evidence of small-bowel strictures, impaction of an intact capsule led to ileus and emergency surgery. Similarly, in the study of Delvaux *et al*[15], of all 22 patients with known or suspected stenosis, the patency capsule induced a symptomatic intestinal occlusion in three patients, which was resolved spontaneously in one and required emergency surgery in two. It was shown that the start of dissolution at 40 h after ingestion was too late to prevent intestinal occlusion. Furthermore, the patency capsule can not detect stenosis and the etiology of small bowel obstruction.

Capsule retention has been defined as the presence of a capsule in the body for a minimum of 2 wk after ingestion, or when the capsule is retained in the bowel lumen indefinitely, unless targeted medical endoscopy or surgical intervention is initiated[16]. In our series, capsule retention occurred only in three cases, in which one of the capsules was spontaneously passed the stricture by medical treatment in 6 mo, and the other two retained capsules were retrieved at surgery. No acute small bowel obstruction occurred after administration of CE. The reported rate of acute abdomen induced by capsule is low. However, there is a controversy in the literature about the utility of capsule retention. In many studies, patients with a high risk of intestinal stricture were excluded for fear of capsule retention, which may have led to acute intestinal obstruction or surgical emergency. However, in most cases, capsule retention is symptomatic, although some patients accepted surgical therapy, which is safe and identifies or treats the underlying disease. Thus, some authors consider that capsule impaction is a valuable means of detecting significant stenosis that would benefit from

surgical management[5]. In addition, the retained capsule can be retrieved using double-balloon endoscopy[17,18]. Importantly, it is necessary to make the patients aware of the potential need for surgery before CE, although the risk for retention was low.

Based on our results, the most common etiology of small bowel obstruction was CD, followed by tumor. In our selected population, capsule retention was asymptomatic, which did not lead to surgical emergency. It is concluded that CE is a safe and effective tool for detecting etiology and stenosis of patients who have a history of small bowel obstruction, especially for the patients with suspected intestinal tumors or CD, which are not identified by routine examinations. Such results need future confirmation from prospective randomized studies.

COMMENTS

Background

Capsule endoscopy (CE) has been demonstrated to be superior to routine radiological examinations in the investigation of obscure gastrointestinal bleeding or suspected Crohn's disease (CD). Small bowel obstruction or strictures are considered to be a contraindication for CE in many centers. However, the accuracy of radiography in this situation has often been questioned.

Research frontiers

CE is now commonly performed for gastrointestinal bleeding of obscure origin or suspected CD. It is noted that the visualization of patients with suspected small bowel stenosis using traditional radiological methods is associated with high false-negative results and radiation doses. Recently, CE or patency capsule has been performed for suspected intestinal strictures in some studies, mainly for patients with known or suspected CD. However, reports about patients with subacute intestinal obstruction receiving CE are rare in the literature up till now.

Innovations and breakthroughs

At many centers, CE is considered to be contraindicated in suspected obstructive small bowel disease, for fear of capsule retention. In the present study, capsule retention occurred only in three cases (one of the capsules was spontaneously passed the stricture by medical treatment in 6 mo, the other two were retrieved at surgery), and no acute small bowel obstruction occurred after administration of CE. CE can be helpful in diagnosing subacute intestinal obstruction in patients with otherwise negative imaging studies, especially for patients with suspected intestinal tumors or CD.

Applications

CE is helpful in diagnosing subacute intestinal obstruction in patients with negative or uncertain imaging studies, which may become an appropriate indication for performing CE.

Terminology

Subacute small bowel obstruction is diagnosed in patients who present with symptoms consistent with small bowel obstruction, in whom abdominal X-ray shows incomplete intestinal obstruction. CE is a novel diagnostic technique that has been used increasingly for analysis of many disorders of the small intestine, such as occult gastrointestinal bleeding, suspected CD, chronic diarrhea, and protein-losing enteropathy.

Peer review

This paper describes CE in patients with small bowl obstruction. Although today the results of MRI of the intestine mostly offers the best chance of finding stenosis, CE is an interesting technique and should lead to a higher percentage of diagnosis.

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BRIEF ARTICLES

Effect of two-channel gastric electrical stimulation with trains of pulses on gastric motility

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Abstract

AIM: To investigate the effect of two-channel gastric electrical stimulation (GES) with trains of pulses on gastric emptying and slow waves.

METHODS: Seven dogs implanted with four pairs of electrodes and equipped with a duodenal cannula were involved in this study. Two experiments were performed. The first experiment included a series of sessions in the fasting state with trains of short or long pulses, each lasted 10 min. A 5-min recording without pacing was made between two sessions. The second experiment was performed in three sessions (control, single-channel GES, and two-channel GES). The stimulus was applied *via* the 1st pair of electrodes for single-channel GES (GES *via* one pair of electrodes located at 14 cm above the pylorus), and simultaneously *via* the 1st and 3rd channels for two-channel GES (GES *via* two pairs of electrodes located at 6 and 14 cm above the pylorus). Gastric liquid emptying was collected every 15 min *via* the cannula for 90 min.

RESULTS: GES with trains of pulses at a pulse width of 4 ms or higher was able to entrain gastric slow waves. Two-channel GES was about 50% more efficient than single-channel GES in entraining gastric slow waves. Two-channel but not single-channel GES with trains of pulses was capable of accelerating gastric emptying in healthy dogs. Compared with the control session, two-channel GES significantly increased gastric emptying of liquids at 15 min (79.0% ± 6.4% *vs* 61.3% ± 6.1%, $P < 0.01$), 30 min (83.2% ± 6.3 % *vs* 68.2% ± 6.9%, $P < 0.01$), 60 min (86.9% ± 5.5 % *vs* 74.1% ± 5.9%, $P < 0.01$), and 90 min (91.0% ± 3.4% *vs* 76.5% ± 5.9%, $P < 0.01$).

CONCLUSION: Two-channel GES with trains of pulses accelerates gastric emptying in healthy dogs and may have a therapeutic potential for the treatment of gastric motility disorders.

Key words: Gastric electrical stimulation; Gastric slow waves; Gastric emptying; Gastrointestinal motility; Gastric pacing

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INTRODUCTION

Gastrointestinal functional or motor disorders are common, affecting 25% of the United State population[1,2] and 5%-10% of Asian population[3]. The patients often complain of a series of dyspeptic symptoms such as nausea and vomiting[4]. Gastric dysrhythmia has been observed in a variety of gastrointestinal motility disorders, including unexplained nausea and vomiting[4], gastroparesis[4,5], type II diabetes[2,5], early pregnancy[6], gastroesophageal reflux disease[7], after vagotomy and surgery[8] or after bone marrow or stem cell transplant[9]. In these circumstances, the frequency of gastric slow wave becomes either abnormally high (tachygastria) or low (bradygastria). Gastric emptying is delayed in

patients with gastroparesis and in about 30%-65% of patients with functional dyspepsia.

The commonly used medical therapy for gastroparesis is prokinetic agents, such as metoclopramide, cisapride, domperidom and erythromycin[10]. However, there are a considerable number of patients who are refractory to these medical therapeutic agents and side effects also limit their usage. While a number of studies have shown that some prokinetics have anti-dysrhythmic effects, but none of them has been developed for the normalization of gastric dysrhythmia[5,11].

Gastric electrical stimulation (GES) or pacing has been under investigation as a potential therapy for gastrointestinal motility disorders[12,13]. A number of studies have been performed to investigate the effect of gastric pacing, but the majority of them seem to indicate that gastric pacing is able to entrain gastric slow waves[14-20], accelerate gastric emptying in patients with gastroparesis[21,22] or in animal model of gastroparesis[18-20]. Three distinct methods have been used in GES, including long pulse stimulation, short pulse stimulation, and stimulation with trains of short or long pulses. In long-pulse stimulation, the pulse width is in the order of milli-seconds and the stimulation frequency is usually in the vicinity of the physiological frequency of gastric slow wave[23-25]. In short pulse stimulation, the pulse width is substantially shorter and is in the order of a few hundred micro-seconds. The stimulation frequency is usually a few times higher than the physiological frequency of gastric slow wave[12,26]. It has been reported that long-pulse stimulation can normalize gastric dysrhythmia, entrain the slow wave[13,17,25,27,28], accelerate gastric emptying in human beings and dogs, and short-pulse stimulation is effective against nausea and vomiting with no or little effect on gastric dysrhythmia, slow waves, or gastric emptying[18,26]. Trains of pulses are composed of a repetitious train of pulses and are derived from the combination of two signals: a continuous signal with a high frequency (in the order of 5-100 Hz) and a control signal to turn the pulses on and off, such as x seconds "on" and y seconds "off". This kind of stimulation has been frequently used in electroacupuncture[29]. Most previous studies were performed using long- or short-pulse GES in patients and in animal model of gastroparesis. Commercially available implantable stimulators are capable of generating short pulses or trains of pulses but not long pulses that are technically difficult to produce. That is, long pulse GES is practically not feasible or much less feasible than GES of pulse trains and has to be replaced by GES with trains of pulses. Accordingly, it is important to study whether the GES with trains of pulses is able to mimic the functions of long pulse GES. However, to the best of our knowledge, few studies have investigated the effect of GES with trains of pulses on gastric motility, such as gastric slow waves and gastric emptying.

This study was to investigate the effect of GES with trains of pulses on gastric slow waves and gastric emptying in health dogs.

MATERIALS AND METHODS

Animal preparation

Seven healthy female beagle dogs, weighing 14-21 kg, were used in this study. After an overnight fasting, the dogs were anesthetized with 2% sodium thiopental (0.6 mL/kg, intravenous) and underwent abdominal surgery. Their tongue color, pulse rate and breath rate were monitored. Four pairs of stainless steel cardiac pacing wires were implanted on the serosal surface of stomach in an arching line along the greater curvature. The most distal pair was placed 2 cm above the pylorus, and the distance between adjacent pairs of electrodes was 4 cm. The bipolar electrodes in each pair were 0.5 cm apart. The electrodes were affixed to the gastric serosa with an unabsorbable suture in the seromuscular layer of stomach. The wires were brought out through the anterior abdominal wall, channeled subcutaneously along the left side of the trunk, and placed outside the skin for pacing or recording gastric myoelectric activity. Each dog was equipped with a duodenal cannula 20 cm beyond the pylorus for the assessment of gastric liquid emptying. The study was initiated after the dogs were completely recovered from surgery, usually 2 wk after surgery. The Animal Care and Use Committee of the Union Hospital of Tongji Medical College approved the surgical and experimental protocols.

Experimental protocol

The study was composed of two experiments using the following protocols. Experiment 1 was designed to assess the optimal stimulation parameters (lowest stimulation energy) to entrain gastric slow waves in the dogs. The dogs were fasted overnight and received no medication before the study. A 30-min baseline recording was made *via* all electrodes in the stomach. Then, an adjustable multi-channel electrical stimulator (model A300, World Precisio Instruments, Sarasota, Florida) was used for stimulation in a constant current mode, and the stimulus consisted of periodic trains of bipolar pulses with adjustable pulse widths. In order to get effective pacing parameters for the entrainment, a series of sessions with various pacing parameters were performed in the fasting state, 10 min each session. A 5-min recording without pacing was made between two consecutive pacing sessions. The pulse width was gradually increased (0.3 ms, 0.5 ms, 0.7 ms, 0.9 ms, 1 ms, 2 ms, 3 ms, 4 ms …) until entrainment of gastric slow waves was achieved. Other parameters for GES were fixed. The stimulus was delivered *via* the 1st channel for single-channel GES (a train on-time of 3 s and off time of 8 s, a pulse frequency of 30 Hz, an amplitude of 5 mA) or *via* both the 1st and 3rd channels for two-channel (channel one: 3 s-on and 8 s-off, 30 Hz, 2 mA; channel three: the same as channel one except for pulse amplitude of 1.6 mA). With this setting, the frequency of pulse train was about 5.5 trains/min, which is similar to the physiological frequency of gastric slow wave. Time delays among different channels were determined

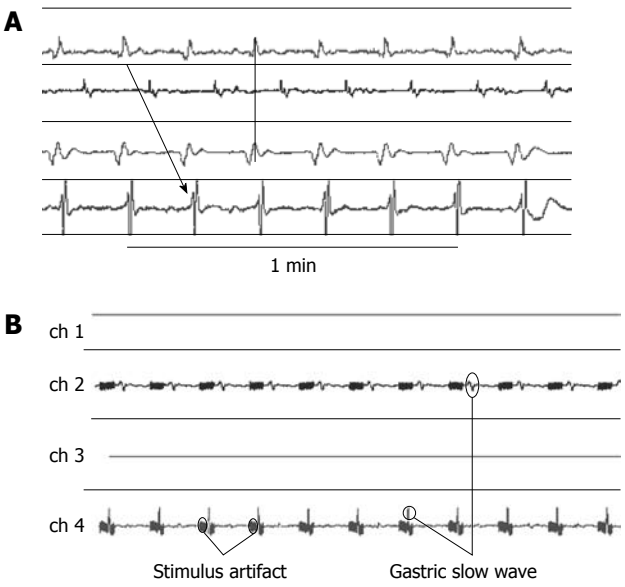

Figure 1 Gastric slow waves at the baseline and with two-channel GES (A), and recordings of gastric slow waves at baseline during 2-channel GES *via* the first and third channels (B).

based on the propagation speed of intrinsic gastric slow waves during the baseline recording. The peak of slow waves occurred simultaneously at the 1st and 3rd channels (a phase shift of 360 degree). Accordingly, stimulation applied in these two channels was synchronic or simultaneous (Figure 1).

Experiment 2 was to investigate the effect of two-channel GES with trains of pulses on gastric emptying. The stimulation parameters were determined as in experiment 1. The study was performed in three sessions (control, single-channel GES, two-channel GES) on three separate days (at least 3 d apart) in a randomized order. Each session consisted of four consecutive 30-min periods of gastric slow wave recordings. During each study session, the dogs were fed with a liquid meal composed of 43 g Nutrison (Nutricia, Holland) and 100 mg phenol red mixed with 100 mL water, immediately after a 30-min baseline recording in the fasting state (the dogs were fasted for 12 h or more). The total volume was 237 mL with a total energy of 250 kcal (6 g fat, 40 g carbohydrate, and 9 g protein). The emptied chyme containing gastric secretion and the ingested liquid meal were collected every 15 min *via* the intestinal cannula for 90 min. The collected volume and the amount of phenol red in each collection were used for the assessment of gastric emptying. Session two was the same as session one, except that GES was performed *via* the 1st channel during the entire postprandial period (Figure 2) with a train on-time of 3 s and off time of 8 s, a pulse frequency of 30 Hz, an amplitude of 5 mA, and width of 4 ms (the optimal pulse width obtained from experiment 1). Session three was the same as session two, except that GES was performed *via* channels one and three (pulse amplitude of 2 mA for channel one and 1.6 mA for channel three). The reduced pulse amplitude in the distal (channel three) stimulation channel was designed to avoid retrograde propagation of stimulation.

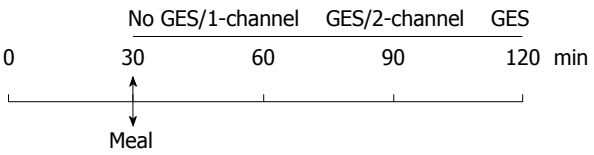

Figure 2 Experiment protocol.

Recording and assessment of gastric slow waves

A multi-channel recorder (AcqknowledgeIII, EOG 100A, Biopac System, Inc. Santa Barbara, CA) was used to record gastric slow waves *via* the serosal electrodes during the entire study. All signals were displayed on a computer monitor and saved on the hard disk with an IBM-compatible 486PC. The low and high cutoff frequencies of the amplifier were 0.05 and 35 Hz, respectively. The most distal recording was used to identify whether gastric slow waves are entrained with GES. Complete entrainment was defined as the frequency of gastric slow waves that was the same as the pacing frequency and phase-locked with the pacing stimulus. The percentage of entrainment of gastric slow waves was defined as the ratio of difference between the recorded slow wave frequency during pacing (f) and the intrinsic frequency before pacing (fi), and the difference between the pacing frequency (fp) and the intrinsic frequency before pacing. It was represented as % of entrainment = (f - fi)/(fp - fi)[28].

Gastric emptying

The test liquid meal contained 100 mg of phenol red as a marker, and gastric emptying was determined by assessment of the amount of phenol red in each collection of gastric effluent as previously described[19]. During the study, the volume of each collection was recorded and a sample of 5 mL was taken and stored in a freezer. At the end of study, these samples were analyzed using a spectrophotometer to detect the amount of phenol red in each sample. Gastric emptying was assessed by calculating the amount of phenol red recovered from each collection of gastric effluent.

Statistical analysis

Results were reported as mean ± SE. The analysis of variance (ANOVA) was used to assess the difference in three sessions of gastric emptying. Paired Student *t* test was used to investigate the differences in gastric emptying between the stimulation and control sessions. $P < 0.05$ was considered statistically significant.

RESULTS

Effect of GES with trains of pulses on gastric slow waves

Gastric slow waves were entrained in each dog with single-channel or two-channel GES using trains of pulses with a greater pulse width. The relation between the entrainment of slow waves and stimulation pulse width is presented in Figure 3. The percentage of entrainment of gastric slow waves with single- or two-channel GES

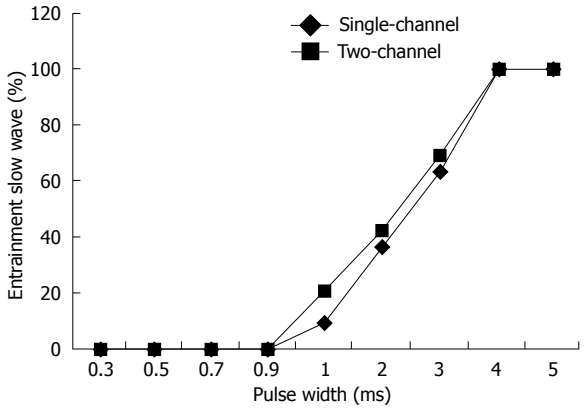

Figure 3 Percentage of slow wave entrainment with GES as a function of stimulation pulse width.

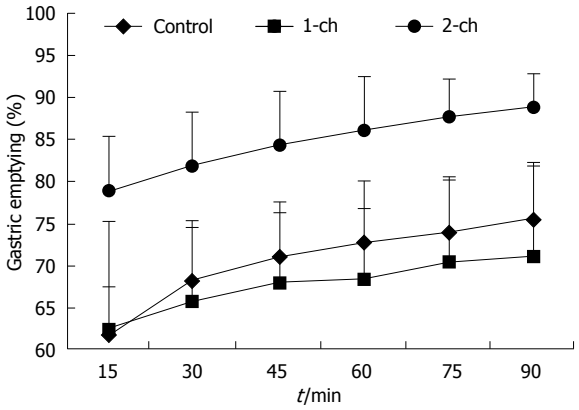

Figure 4 Gastric emptying in the control sessions and sessions with single-channel or two-channel GES. $P < 0.01$ (2-ch *vs* control).

was 7.75% ± 1.62% or 19.25% ± 9.04% at 1 ms, 36.62% ± 5.75% or 42.82% ± 5.45% at 2 ms, 62.34% ± 7.38% or 67.75% ± 9.80% at 3 ms and 100% ± 0% or 100% ± 0% at 4 ms, respectively. A complete entrainment was achieved with GES with a pulse width of 4 ms or greater. Some typical recordings at the baseline and during GES are shown in Figure 1. The entrainment of gastric slow waves usually occurred a few minutes after gastric pacing as demonstrated by the fact that the slow waves were phase-locked with the pacing stimulus a few minutes after the initiation of pacing.

To compare the stimulation energy required to completely entrain the gastric slow waves, the following formula was used for the calculation of stimulation energy (E): E = (cycles/min) × (frequency) × (pulse width) × (amplitude)^2. Accordingly, the minimum energy required by single-channel GES was 16 500 ms × mA^2, whereas that for the two-channel GES was 8421.6 ms × mA^2, which represents about 51.04% of the energy required by single-channel GES or a saving of 48.96% of energy.

Effect of GES with trains of pulses on gastric emptying

Two-channel GES with trains of long pulses (pulse width: 4 ms) could accelerate gastric emptying in the healthy dogs ($P < 0.01$, ANOVA) (Figure 4). Compared with the control session, two-channel GES significantly increased gastric emptying of liquids at 15 min (79.0% ± 6.4% *vs* 61.3% ± 6.1%, $P = 0.001$), 30 min (83.2% ± 6.3 % *vs* 68.2% ± 6.9%, $P = 0.005$), 60 min (86.9% ± 5.5% *vs* 74.1% ± 5.9%, $P = 0.010$), and 90 min (91.0% ± 3.4% *vs* 76.5% ± 5.9%, $P < 0.0037$), respectively, after feeding. However, no significant difference was noted in gastric emptying between single-channel GES and control sessions.

DISCUSSION

In the present study, GES with trains of wider pulses (width ⩾ 4 ms) but not short pulse could entrain gastric slow waves. Two-channel GES but not single-channel GES, significantly accelerated gastric emptying of liquids in healthy dogs.

Most previous studies showed that long pulse GES can entrain gastric slow waves in human beings and animals[19,20,22-25,27]. None of these studies has investigated the effect of GES with trains of pulses on gastric slow waves. It has been shown that GES with trains of short pulses can improve symptoms, such as nausea and vomiting of patients with gastroparesis[24,26], but cannot entrain gastric slow waves or normalize gastric dysrhythmia. In this study, GES with trains of pulses entrained gastric slow waves as long as the width of pulses in the train was 4 ms or greater. The energy required to completely entrain gastric slow waves with two-channel GES was less than that with single-channel GES, which might be due to the fact that each stimulation was responsible for entraining slow waves in a smaller region (about 50%) of the stomach with two-channel GES, compared with single-channel GES.

Conventionally, long-pulse GES is performed using a single pair of electrodes or single-channel GES. It has been reported that single-channel GES with long pulses accelerates gastric emptying in patients with gastroparesis[22] and in animal models of gastroparesis[30], and has no effect on gastric emptying in healthy dogs[18,19,30]. Recent studies on the effect of multi-channel GES on gastric emptying and entrainment of slow waves indicate that multi-channel stimulation with long pulses is more efficient than single-channel stimulation for the entrainment of slow waves, and can accelerate gastric emptying[18-20,31]. It has been shown that four-channel long pulse GES can accelerate gastric emptying in healthy dogs[18], whereas two-channel long pulse GES can normalize vasopressin-induced delayed gastric emptying in dogs[19]. To date, no study is available on the effect of multi-channel GES with trains of pulses on gastric emptying. In the present study, we used single-channel (14 cm above the pylorus) and two-channel (6 and 14 cm above the pylorus) GES to investigate their effect on gastric emptying. Compared with the control session, two-channel but not single-channel GES with trains of pulses significantly accelerated gastric emptying, which is consistent with the previous findings.

It is well known that gastric emptying of liquid and solid occurs separately, involving different areas

of stomach. It is believed that the antrum undergoes orderly peristaltic contractions and acts as a pump, while the proximal segment functions as a reservoir[32]. Gastric emptying demands accommodated motion of the proximal and distal stomach. The motility of stomach follows an orderly pattern in which gastric peristaltic contractions are phase-locked with gastric pacesetter potentials, which sweep distally from the corpus toward the pylorus. It is also known that gastric contractions are controlled by gastric slow waves. Multi-channel GES more accurately mimics the natural propagation and characteristics of gastric slow waves[18,30], thus controlling gastric contractions more effectively.

In this study, two-channel GES with trains of pulses entrained gastric slow waves and accelerated gastric emptying in healthy dogs, suggesting that two-channel GES with trains of pulses might be applicable in treatment of gastroparesis and normalization of gastric dysrhythmia. Technically, it is more feasible to make an implantable stimulator using trains of pulses than using repetitive long pulses due to the current charge balance. Accordingly, GES with trains of pulses is technically more attractive than long pulse GES. Currently, most commercially available implantable stimulators use trains of pulses. However, none of them is able to generate pulses with a width of 4 ms or greater. Therefore, new hardware design and development are needed before two-channel GES with trains of pulses can be used in clinical practice.

In conclusion, entrainment of gastric slow waves is feasible using GES with trains of pulses at a pulse width of 4 ms or greater. Two-channel GES with trains of pulses can accelerate gastric emptying in healthy dogs and may have a therapeutic potential for the treatment of gastric motility disorders.

COMMENTS

Background

Gastric dysrhythmia and delayed gastric emptying have been observed in a variety of gastric motility disorders. Treatment options for such disorders include medical therapy, surgical therapy, and nutritional support.

Research frontiers

Gastric electrical stimulation (GES) or pacing has been under investigation as a potential therapy for gastrointestinal motility disorders. However, few studies are available on the effect of two-channel GES with trains of pulses on gastric slow waves and gastric emptying.

Innovations and breakthroughs

In this study, the authors used single-channel (14 cm above the pylorus) and two-channel (6 and 14 cm above the pylorus) GES to investigate their effect on gastric emptying. Compared with the control session, two-channel but not single-channel GES with trains of pulses significantly accelerated gastric emptying, which is consistent with the previous findings.

Applications

Two-channel GES could entrain gastric slow waves and accelerate gastric emptying in healthy dogs, suggesting that two-channel GES with trains of pulses can be used in treatment of gastroparesis and normalization of gastric dysrhythmia.

Terminology

GES with trains of pulses, composed of a repetitious train of pulses, is derived from the combination of a continuous pulse singal with a high frequency (in the order of 5-100 Hz) and a control signal to turn the pulses on and off, such as x seconds "on" and y seconds "off". This kind of stimulation has been used in electroacupuncture.

Peer review

The authors have demonstrated that entrainment of gastric slow waves is feasible with GES of trains of pulses at a pulse width of 4 ms or greater and two-channel GES with trains of pulses can accelerate gastric emptying in healthy dogs, thus having a therapeutic potential for the treatment of gastric motility disorders.

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CASE REPORT

Adult hereditary fructose intolerance

Mohamed Ismail Yasawy, Ulrich Richard Folsch, Wolfgang Eckhard Schmidt, Michael Schwend

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Author contributions: Yasawy MI was responsible for the fructose tolerance test, analysis of the references obtained from literature search and final write-up of the paper; Folsch UR offered the case; Schmidt WE was responsible for DNA test; Schwend M was responsible for literature search and collected the relevant references related to the case.
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Abstract

Hereditary fructose intolerance (HFI) is an under-recognized, preventable life-threatening condition. It is an autosomal recessive disorder with subnormal activity of aldolase B in the liver, kidney and small bowel. Symptoms are present only after the ingestion of fructose, which leads to brisk hypoglycemia, and an individual with continued ingestion will exhibit vomiting, abdominal pain, failure to thrive, and renal and liver failure. A diagnosis of HFI was made in a 50-year-old woman on the basis of medical history, response to IV fructose intolerance test, demonstration of aldolase B activity reduction in duodenal biopsy, and molecular analysis of leukocyte DNA by PCR showed homozygosity for two doses of mutant gene. HFI may remain undiagnosed until adult life and may lead to disastrous complications following inadvertent fructose or sorbitol infusion. Several lethal episodes of HFI following sorbitol and fructose infusion have been reported. The diagnosis can only be suspected by taking a careful dietary history, and this can present serious complications.

Key words: Adults; Fructose intolerance; Diet; Fructose; Sorbitol

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INTRODUCTION

Hereditary fructose intolerance (HFI) is an autosomal recessive inborn error of metabolism that results from a deficiency of fructose 1-phosphate aldolase in the liver, intestine and kidney.

The estimated incidence is 1 in 20 000 live births[1] and the carrier frequency is 1 in 70, but the prevalence of HFI in adults is unknown. The clinical symptoms were first described by Chambers and Pratt in 1956[2].

Affected individuals fail to metabolize fructose completely in the liver, intestine and kidneys because of deficiency of fructose 1-phosphate aldolase and ingestion of fructose, sorbitol or sucrose causes abdominal pain, vomiting and symptomatic hypoglycemia. The syndrome typically appears in the newborn at the time of weaning from the breast when food containing sucrose or fructose is given. Continued ingestion results in poor feeding, growth retardation, gradual liver and kidney failure acidosis, and eventually death[3]. Affected children soon develop an aversion to all foods and protect themselves by self-imposed fructose and sucrose restriction.

The strict dietary exclusion leads to normal growth and longevity. Nevertheless, complete elimination of this sugar from the diet is difficult to achieve, especially for undiagnosed adults, without professional advice. These people may suffer symptoms throughout life and represent a diagnostic challenge for attending physicians. Furthermore, potentially lethal complications may result from inadvertent infusion of fructose- or sorbitol-containing solutions in a hospital setting[4,5].

CASE REPORT

A 50-year-old German woman presented with a long life history of aversion to sugary foods. She reported being breast fed until the age of 2 years, and her mother said that she refused the usual sucrose-containing formulas. She described nausea, vomiting, diffuse abdominal pain and hypoglycemic symptoms even after the smallest amount of sugar or fruit. Her 2-year-old brother died after receiving an intravenous infusion in hospital, while her parents and three siblings are asymptomatic. She takes no regular medications. On examination, she

Table 1 FTT using 250 mg/kg body weight showing glucose, phosphate, uric acid and magnesium levels in the blood following injection of fructose

Time (min)	Glucose (mg/dL)	Phosphate (mmol/L)	Uric acid (mg/dL)	Magnesium (mmol/L)
0	87	1.000	5.1	0.72
15	67	0.77	6.74	0.84
30	61	0.64	7.74	0.8
45	60	0.80	7.58	0.88
60	68	0.83	7.58	0.91

had mild thoracic scoliosis with no neurological defect. Otherwise, physical examination was unremarkable.

Results of laboratory investigations including full blood count, urea, creatinine, electrolytes, full biochemical profile, amylase lipase and lipid studies, liver function tests and insulin level were within normal ranges. A fructose tolerance test (FTT) using 250 mg fructose per kilogram body weight was performed. At 0, 15, 30, 45 and 60 min after fructose injection, blood samples were taken for analysis of glucose, phosphate, uric acid and magnesium. A typical abnormal FTT was observed after the infusion, i.e. a drop in serum glucose and serum phosphate and rise in serum uric acid and magnesium concentration occurred (Table 1).

Thirty minutes after fructose injection, she developed significant dizziness, sweating, tremor and abdominal pain that were closely observed, and by 60 min her symptoms improved. The diagnosis was further confirmed by histochemical analysis of an endoscopic biopsy specimen from the small intestine, which showed 70% reduction in aldolase B activity in the mucosa, and molecular analysis of leukocyte DNA extracted from a blood sample using PCR amplification revealed that she had inherited two doses of the mutant gene, one from each parent, as the cause of the disease.

DISCUSSION

Fructose is a natural component of many plants and is distributed widely among most fruits and vegetables. Fructose is metabolized primarily in the liver and to some extent in the kidney, small intestine and adipose tissue[6]. Deficiency in aldolase B in the liver, kidney and small intestine causes fructose intolerance[7]. After ingestion, fructose rapidly enters the hepatocytes where fructokinase phosphorylates it to fructose 1-phosphate. Fructose 1-phosphate accumulates in HFI because of deficiency of the enzyme fructose 1-phosphate aldolase, which splits fructose 1-phosphate into glyceraldehydes and dihydroxyacetone phosphate.

The accumulation of fructose 1-phosphate results in inhibition of other enzymes, namely phosphorylase, liver fructose 1-6 bisphosphate aldolase and fructokinase. This results in impaired glycogenolysis and glyconeogenesis, and may induce hypoglycemia[8]. Early exclusion of fructose and sucrose from the diet is accompanied by dramatic improvement; otherwise, growth is retarded and progressive liver and renal disease are likely, and may lead to death[9-11]. Diagnosis can be achieved by FTT and tissue diagnosis by direct assay of aldolase B activity in the liver, intestine or renal tissue. Recently, the use of PCR-based procedures has made the diagnosis simpler[12,13].

The infusion of fructose- or sorbitol-containing solutions in patients with unsuspected disease leads to potentially fatal hepatorenal failure. More than 20 cases have been reported in Germany where the use of fructose or sorbitol solutions is long established[14,15]. Our patient is alive at the age of 50 years with previously undiagnosed HFI, and did not have complications of the disease. This patient illustrates the importance of a careful dietary history and awareness of disease symptoms. In contrast, incorrect diagnosis and unawareness of possible pediatric problems in adult life may lead to catastrophic complications, while early recognition leads to effective management.

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CASE REPORT

Drug-induced liver injury due to "natural products" used for weight loss: A case report

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Abstract

Taking herbal-extracts to lose weight is an underestimated health hazard. Often, these products contain active agents that can cause acute liver damage. In this case report, a 22-year-old female patient, who presented with a feature of cholestatic syndrome, was so sure that the "natural products" were not dangerous that she did not inform her physicians that she had taken them, making their task that much more challenging. Clinical presentation mimicked acute cholecystitis and the patient underwent a cholecystectomy. Surgery was without any consequences and complications, although it did not completely cure the illness. She later admitted to having taken herbal remedies and this led to the correct diagnosis of phytotherapy-related hepatotoxicity and a successful therapeutic approach. The true incidence of phytotherapy-related hepatotoxicity and its pathogenic mechanisms are largely unknown. It is important to increase the awareness of both clinicians and patients about the potential dangers of herbal remedies.

Key words: Drug-induced liver injury; Obesity; Herbal remedies; Cholecystitis

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INTRODUCTION

The desire to lose weight using "natural products" and the availability of these items can induce pathologies, the causes of which are often overlooked. The main problem with "natural products" is that the exact quantity and purity of a given ingredient contained in extracts of vegetable origin (mainly herbs) are largely unknown. It can happen that patients deny taking these products, thinking that they are "safe" because they are "natural" and thus physicians do not have the key facts to interpret dangerous pathologies. Here we report a case of a patient who experienced jaundice and pruritus, not assuming the "natural products" was at fault, with a clinical presentation highly suggestive of cholecystitis whose final diagnosis turned out to be drug-induced liver injury (DILI).

CASE REPORT

A 22-year-old obese (BMI 32) woman presented to hospital in May 2007 with a cholestatic syndrome of unknown origin. Her only declared pre-existing medication was paracetamol (500 mg daily), used as an analgesic for menstrual pain. Pre-admission blood tests were normal.

Symptoms included jaundice, pruritus, right upper quadrant pain and epigastric tenderness, accompanied

by fever of low grade, nausea, vomiting, dark urine and pale stools. At the time of admission routine liver enzyme tests revealed bilirubin 128 μmol/L (< 20 μmol/L), alkaline phosphatase (ALP) 1229 U/L (40-110 U/L), γ-glutamyltransferase (GGT) 293 U/L (< 50 U/L), Aspartic-aminotransferase (AST) 1378 U/L (< 45 U/L) and Alanine-aminotransferase (ALT) 1686 U/L (< 40 U/L). She had never consumed alcohol and there was no recent travel history. Viral serology for hepatitis and HIV were negative. An infection screen was carried out because our country has an increasing incidence of exotic illnesses due to recent immigration that can cause transient liver enzyme derangement. This panel included cytomegalovirus, Epstein-Barr virus, Flavivirus, Dengue virus, Ross River virus, Barmah Forest virus, Spotted fever virus, Scrub Typhus, and Leptospirosis serology; all of them were unremarkable. Serum copper and caeruloplasmin, α-fetoprotein, α-1 antitrypsin, iron deposits were in the normal range. Anti-nuclear antibodies, perinuclear antineutrophil cytoplasmic antibodies, antibodies to liver kidney microsomal antigen type-1, anti-mitochondrial and anti-smooth muscle antibodies were negative. Her complete blood count was slightly abnormal. In fact, a modest increase in WBCs without left shift was present on admission, concurrent with an increase in eosinophils (7%) and a decrease in lymphocyte counts.

Abdominal ultrasound revealed the presence of microcalculi in the gallbladder. No clear dilatation of the common bile duct was seen, but the exam was performed in the presence of marked abdominal meteorism.

A magnetic resonance cholangio-pancreatography (MRC) indicated a dilatation of the choledocus with a likely interruption of its terminal tract, with some evidence of microstones in the gallbladder (Figure 1). Consequently, physicians empirically treated this illness by imipenem/cilastatin (500/500 mg every 6 h) for seven days.

A negative history of drug use, the physical findings (i.e. right upper quadrant and epigastric tenderness in the absence of peritoneal findings), laboratory data (i.e. elevated levels of bilirubin, alkaline phosphatase, ALT, and γ-glutamyltransferase) were consistent with extrahepatic obstruction, suggesting stones complicated by acute cholecystitis. Therefore, to gain access to and/or remove impacted common bile duct (CBD) stones at the ampulla of Vater, the patient was submitted to a preoperative endoscopic retrograde cholangiopancreatography (ERCP) with endoscopic sphincterotomy and extraction of sand-like stones.

Using this standard therapy, her laboratory data improved, with AST and ALT values of 1110 U/L and 1225 U/L, respectively. At this point, a laparoscopic operative CBD exploration with mini-invasive technique was planned.

The abdominal inspection showed the presence of extensive visceral adherences, as well as a general aspect of diffuse bowel inflammation. The gallbladder was reddish-colored with a thick wall and bled easily. This was suggestive of a complex abdominal pathology, therefore a conversion to an open procedure was chosen.

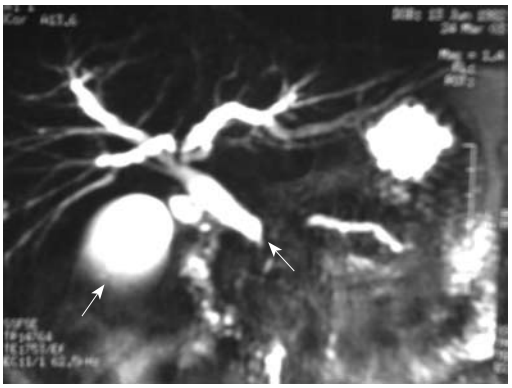

Figure 1 Magnetic resonance cholangio-pancreatography showing dilatation of the choledocus and mimicking an interruption of its terminal tract, with some evidence of microstones in the gallbladder (arrows).

Abdominal exploration and biliary manometry caused the surgeons to utilize an ante-grade trans-ampullary intra-operative endobiliary stenting. The macroscopic examination showed acute cholecystitis with moderate wall-thickening containing very dense bile and some small stones.

The postoperative course was pain-free and the patient was prematurely discharged with drastically reduced (though elevated compared to normal) enzymatic values of AST 108 U/L and ALT 156 U/L.

Four days later the patient underwent liver laboratory tests that underlined an elevation of the transaminases, with an AST level of 644 U/L, ALT of 810 U/L, AP of 806 U/L and bilirubin of 64 μmol/L, mainly unconjugated with a persistently draining T-tube.

An interrogation of the woman's relatives highlighted that the patient had taken several doses of a phyto-preparation in solution, bought from a store run by an herbalist, labeled as "herbal therapy for losing weight". Fortunately, the relatives found this preparation, containing "Lycopodium serratum and Chelidonium maius" at home. The Roussel Uclaf Causality Assessment Method, also known as Danan's international consensus criteria[1], was developed to quantify the strength of association between liver injury and herbal remedies and implicated the phyto-preparation as causing the injury. The case was adjudicated by three reviewers (DC, GT, MND) working independently and the patient was diagnosed as likely to be suffering from DILI. One month after given up taking the herbal remedy, all the liver parameters returned to normal values, without any apparent consequences. The case was re-adjudicated after a four-week follow-up by the same reviewers and the diagnosis was confirmed.

DISCUSSION

The "chelidonium majus" belongs to the family of papaveraceae; its roots contain the biologically active components chelerythrine and sanguinarine. The active "principia" are similar to those of opium, and have well-known hepatotoxic effects[2-4], although in animals an average daily oral dose of alkaloids up to 5 mg/kg has

been proven to be safe[5].

The herb "*Lycopodium serratum*" has several[6] active agents that can cause hepatotoxicity[7]. The hepatic damage caused by these agents, generally of a cholestatic type, is possibly mediated by an idiosyncratic or hypersensitivity reaction. Recently, a different hypothesis was proposed involving an impairment of mitochondrial respiration[8].

Although this is not the first case reported in the literature, its importance lies in the atypical presentation. Indeed, was this a case of misdiagnosis, the co-existence of two diseases or an uncommon manifestation of DILI with a clinical presentation mimicking an other disease? The results of liver laboratory tests and imaging studies were attributed to an earlier combination of symptomatic gallstones and cholangitis and the patient was treated accordingly. Unfortunately, a liver biopsy, which would have indicated the presence of canalicular cholestasis with bile plugs in dilated canaliculi, occasional portal tracts containing a prominent lymphocytic infiltrate with mild piecemeal necrosis, was not performed and consequently the opportunity for a definitive diagnosis was lost.

Gallstone disease remains one of the most common medical problems. The risk factors predisposing to gallstone formation include obesity, diabetes mellitus, estrogen and pregnancy, hemolytic diseases, and cirrhosis. Acute cholecystitis can carry the risk of complications, including empyema, perforation, abscess, peritonitis and sepsis. Acute cholecystitis also causes acute pain in the right upper quadrant (RUQ). However, cross-sectional imaging is essential, because more than one-third of patients with acute RUQ pain do not have acute cholecystitis. Today, laparoscopic cholecystectomy, laparoscopic common bile duct exploration, and endoscopic retrograde management of CBD stones play important roles in the treatment of gallstones, even though the treatment of choice remains cholecystectomy. However, when asymptomatic gallstones are detected during the evaluation of a patient, a prophylactic cholecystectomy is normally not indicated because of several factors. Only about 30% of patients with asymptomatic cholelithiasis will warrant surgery during their lifetime, suggesting that cholelithiasis is a relatively benign condition in some people.

The main question we should ask ourselves is: was surgery the right choice? Although the patient's symptoms and signs were extremely atypical for establishing the diagnosis of acute cholecystitis in this young immune-competent patient, her declaration of not having taken any other medications, including over-the-counter medications, herbal or traditional medicines, definitely misled physicians. The late admission by her parents allowed a correct diagnosis of DILI and not acute cholecystitis. Given the diagnosis of DILI, the patient took a further risk with anesthesia.

Should physicians have performed further studies before surgery? A CT cholangiogram would have shown contrast material being excreted by the renal tract, suggesting that the pathology concerned hepatocellular damage rather than a biliary obstruction.

Was the patient incautiously discharged from the surgery unit? The answer is probably yes, because the reduction of liver enzymatic activity caused surgeons to underestimate the pathology, with overconfidence in the previous diagnosis of cholecystitis.

There are many examples of hepatotoxicity induced by herbal remedies, which have been widely used in recent decades as weight loss agents. Germander (Teucrium chamaedrys) extracts cause DILI, probably mediated by furano neoclerodane diterpenoids[9]. Chaparral is a desert shrub traditionally used by Native Americans for treatment of several ailments. Recently, preparations of chaparral leaves have been marketed as weight loss agents. The mechanism of chaparral toxicity involves its active ingredient, nordihydroguaiaretic acid[10]. Kava (kava kava, awa, or kew), derived from the dried root and rhizome of Piper methysticum, has recently been marketed as an anxiolytic and mood enhancer. Recent studies from Europe have described cases of kava-associated hepatic injury. The mechanism of hepatic injury appears to be immune-mediated, with CYP2D6 deficiency perhaps being a risk factor[11]. *Herba Ephedrae* (from Ephedra sinica and other Ephedra species) is a traditional Chinese extract also used for treatment of asthma, nasal congestion, and fever. Although most adverse effects of *Herba Ephedrae* are cardiovascular or neurological, 4% of reports mentioned acute hepatitis. *Herba Ephedrae* contains phytochemicals, which are thought to strengthen its toxic activity[12].

In addition to the above supplements, liver injury has been attributed to other botanical agents. The pyrrolizidine alkaloids found in comfrey leaves and Heliotropium, Senecio, and Crotalaria species are known to cause veno-occlusive disease of the liver *via* a toxic effect[13]. Mixtures of valerian and skullcap (Valeriana officinalis and Scutellaria lateriflora) have induced hepatitis *via* alkylating agents. LipoKinetix was marketed as a dietary supplement for weight loss. Hepatic injury appears to be due to an idiosyncratic reaction, perhaps related to phenylpropanolamine[14]. Among other weight loss agents, Usnic acid should be suspected in case of severe hepatotoxicity[15].

In our case, the patient was sure the product was harmless and denied the use of a potentially dangerous product in her history, thus not allowing physicians to discover the etiology of the serious pathology from which she suffered. Only an accurate interrogation of relatives was able to discover the relationship between the herbal remedy and DILI.

The diagnostic approach was, in spite of the lack of a certain etiology, the most cautious possible; in fact, MRC and ERCP, perfectly framed into the clinical picture of this patient, are generally considered investigations of first level.

The prevalence of adverse drug reactions (ADR) in health care systems has generated immense interest in recent years. Some of these adverse events are completely unpredictable, but some result from medical errors, patient's negligence or ignorance, and may occur anywhere and at anytime in the health care processes. However, a majority of them may be preventable. The consequences of these ADRs might vary, from little or

no harm to ultimately being fatal to the patients.

Patient safety has received increased attention in recent years, but mostly with a focus on the epidemiology, rather than on practices that reduce (1) ADRs, (2) adverse events related to exposure to herbal remedies and dietary supplements and (3) invasive procedures in medical care involving a wide spectrum of diagnoses or conditions. Potential safety practices should be identified, based on preliminary surveys of the literature and expert consultation.

The misdiagnosis of DILI has many ramifications. These include medical and psychological implications for patients and their families, and financial and public health implications for health-care institutions.

The patient could have sued the health care practitioners (specifically the surgeons) if she felt she had been injured. However, successful medical malpractice lawsuits require proof of the following items: the care provided was below the ordinary standard of care that would be provided by similar health care practitioners under similar circumstances and the patient was harmed because of the deviation from the standard of care. In our case, concerns about lawsuits did not arise, because the physicians' actions were in the best interests of the patient. In fact, a good defence against malpractice lawsuits is to provide excellent medical care and to build close, trusting, and collaborative relationships with patients.

As a final consideration, patients should be especially cautious about using drugs, and should inform their doctor about any drugs or other substances they are taking, including prescription and over-the-counter medications, recreational drugs, herbal remedies, and nutritional supplements. Health care professionals are encouraged to report all ADRs, especially hepatotoxicity and to pay much more attention in prescribing and administering drugs.

For the vegetal abstracts, it should be mandatory to correctly describe their contents, taking in account the active ingredients, the real quantity per unit of product contained with the preparation, and to make clear any possible side effects.

In conclusion, the authors believe that a detailed, painstaking and meticulous history could have unveiled the underlying condition and the patient would not have been subjected to invasive and potentially harmful interventions. This is probably the most important learning point that emerged from this case report.

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CASE REPORT

Primary hepatic carcinoid: A case report and literature review

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Abstract

Carcinoids are tumors derived from neuroendocrine cells and often produce functional peptide hormones. Approximately 54.5% arise in the gastrointestinal tract and frequently metastasize to the liver. Primary hepatic carcinoid tumors (PHCT) are extremely rare; only 95 cases have been reported. A 65-year-old man came to our attention due to occasional ultrasound findings in absence of clinical manifestations. His previous medical history, since 2003, included an echotomography of the dishomogeneous parenchymal area but no focal lesions. A computed tomography scan performed in 2005 showed an enhanced pseudonodular-like lesion of about 2 cm. Cholangio-magnetic resonance imaging identified the lesion as a possible cholangiocarcinoma. No positive findings were obtained with positron emission tomography. Histology suggested a secondary localization in the liver caused by a low-grade malignant neuroendocrine tumor. Immunohistochemistry was positive for anti chromogranin antibodies, Ki67 antibodies and synaptophysin. Octreoscan scintigraphy indicated intense activity in the lesion. Endoscopic investigations were performed to exclude the presence of extrahepatic neoplasms. Diagnosis of PHCT was established. The patient underwent left hepatectomy, followed by hormone therapy with sandostatine LAR. Two months after surgery he had a lymph nodal relapse along the celiac trunk and caudate lobe, which was histologically confirmed. The postoperative clinical course was uneventful, with a negative follow-up for hematochemical, clinical and radiological investigations at 18 mo post-surgery. Diagnosis of PHCT is based principally on the histopathological confirmation of a carcinoid tumor and the exclusion of a non-hepatic primary tumor. Surgical resection is the recommended primary treatment for PHCT. Recurrence rate and survival rate in patients treated with resection were 18% and 74%, respectively.

Key words: Carcinoid; Primary hepatic carcinoid; Neuroendocrine neoplasm; Therapy; Surgical treatment; Prognosis

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INTRODUCTION

Carcinoids are tumors of neuroendocrine origin, capable of producing functional peptide hormones. The literature has reported different classifications, mainly based on either anatomo-pathological and/or clinical criteria of neuroendocrine tumors of ubiquitous distribution. Several organs may be involved, such as the adrenal gland (pheochromocytoma), the thyroid (midollar carcinoma) and the lung, where microcytoma accounts

for 20%[1] while carcinoids represent 1%-2% of all pulmonary neoplasms in the typical variant (80%-90%) and atypical (10%-20%)[2,3].

About 54.5% of carcinoid tumors arise within the gastrointestinal system and frequently metastasize to the liver[4]. Primary hepatic carcinoid tumor (PHCT) is an extremely rare neoplasm affecting relatively young subjects with an average age of 45 years[5] and no gender predominance. Diagnosis of PHCT is mainly achieved through histological confirmation and exclusion of other sites of the disease[6].

Here, we report the case of an occasional finding of a hepatic lesion, which led to the diagnosis of PHCT after a complicated diagnostic process.

CASE REPORT

A 65-year-old man presented with hypertension, peripheral vascular disease, and statin treated dyslipidemia. His previous medical history, dating back to spring 2003, included an echotomography of the dishomogeneous parenchymal area with no focal lesions. A computed tomography (CT) scan showed no steatosis in the image area. In July 2005, a CT scan of the asymptomatic patient showed an enhanced a pseudonodular-like lesion of about 2 cm localized in hepatic segments Ⅱ-Ⅲ, with intra-hepatic biliary dilatation (Figure 1).

The patient was admitted to hospital for further clinical investigations. Blood chemical analyses showed no abnormalities, not even the presence of markers (CEA, CA 19-9, α FP). The laboratory results are shown in Table 1.

Due to the doubtful interpretations of the radiological findings, a magnetic resonance imaging (MRI) was carried out (Figure 2). The investigation revealed a pseudonodular mass of about 5 cm × 3 cm characterized by low signal intensity both on T1 and T1FS weighted images, as well as weak irregular high signal intensity on T2 and T2FS weighted images. Moreover, an intra-hepatic biliary dilatation was described at the source of the lesion, thus leading us to suspect a heteroplastic lesion similar to a cholangiocarcinoma.

The patient was therefore referred for an 18-fluorodeoxyglucose positron emission tomography (PET), which proved negative. This confirmed by histological examination of a biopsy sample from the lesion. The cytohistological and immunohistochemical picture proved to be consistent with a hepatic localization of a low-grade malignant neuroendocrine carcinoma (presence of anti-chromogranin and Ki 67 antibodies; positive for synaptophysin and S 100 protein) while serum markers were negative (CgA, NSE).

Octreotide scintigraphy using 111 In-pentetreotide (octreotide scan) confirmed the diagnosis as well as enhancing a marked hyperactivity near the lesion in the left hepatic lobe associated with adenopathy in the interaortocaval site (Figure 3A). The patient thus underwent further investigations to exclude metastases with extra hepatic lymph node involvement by endoscopic examinations of the gastroenteric tract (esophagus-gastro-duodenoscopy, colonoscopy, and capsular endoscopy) and analysis of the respiratory systema (bronchoscopy) which all proved negative.

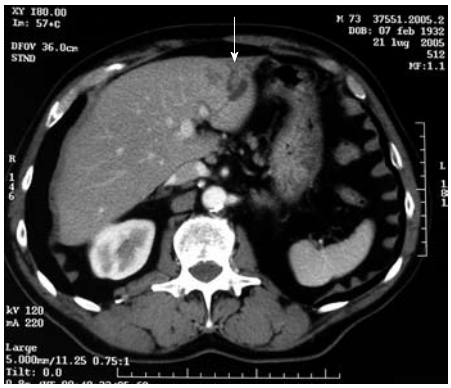

Figure 1 Abdominal CT scan (July 2005) showing a low-density pseudonodular area (arrow) of 2 cm with biliary dilatation.

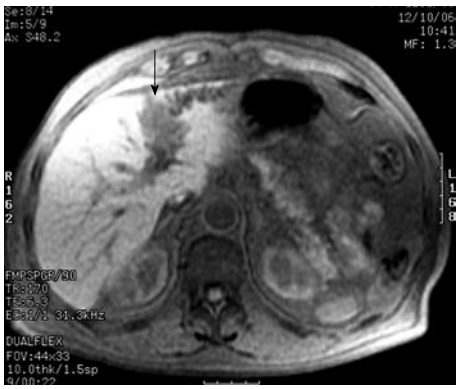

Figure 2 Abdominal MRI (August 2005) showing a pseudonodular mass (arrow) measuring about 5 cm × 3 cm of the hepatic segments Ⅱ-Ⅲ. At the bottom of the lesion, the biliary tree appears dilated.

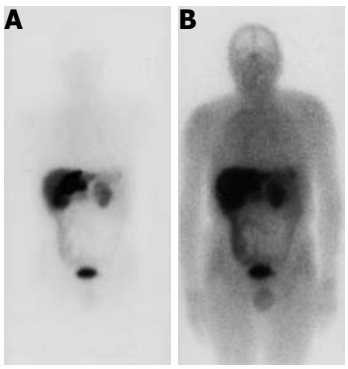

Figure 3 111 In-pentetreotide (octreotide) scintigraphy. A: Before hepatectomy: marked hyperactivity of the lesion is observed in the left hepatic lobe and interaortal adenopathy is observed; B: After hepatectomy; abnormal fluid accumulation of ligand in the epigastric region and dishomogeneous hepatic distribution.

The patient suffered no flushing, no abdominal pain, and no alvus alteration. Moreover, the patient only referred to some successive episodes of angioneurotic edema of the face in the previous 10 years, regardless of the disease in question. As a result, the patient underwent an uncomplicated left hepatic resection and appendectomy with an uneventful postoperative clinical course. Subsequently, octreotide therapy was

Table 1 Hematological values of the patient and normal range

Variable	Patient value	Normal value	Variable	Patient value	Normal value
Erythrocytes	4.93×10^6 U/L	4.2-5.4	Amylase	60 U/L	30-110
Leucocytes	8.16×10^3 U/L	4-10	Total proteins	8.3 g/dL	6.3-8.2
Hemoglobin	14.9 g/dL	12-16	Urea	79 mg/dL	10-50
Platelets	2.22×10^5 U/L	150-400	LDH	250 U/L	313-618
Creatinine	1.1 mg/dL	0.7-1.2	ESR	15 mm/s	1-30
PT	95%	70%-100%	CRP	3 mg/L	up to 3
Total bilirubin	1 mg/dL	0.2-1.3	CEA	2.6 ng/mL	up to 5
GOT	23 U/L	up to 40	CA 19-9	7 ng/mL	up to 37
GPT	22 U/L	9-56	α FP	3 ng/mL	up to 15
GGT	84 U/L	12-58	NSE	8.7 ng/mL	up to 14
ALP	73 U/L	38-126	CgA	72 ng/mL	20-98
Albumin	4.8 g/L	35-52	Anti HCV	Negative	
Cholinesterase	7926 U/L	4650-12220	HbsAg	Negative	
Total cholesterol	258 mg/dL	145-200	Triglycerides	125 mg/dL	50-170

PT: Prothrombin time; GOT: Glutamic oxaloacetic transaminase; GPT: Glutamic pyruvic transaminase; GGT: Gamma glutamyl transpeptidase; ALP: Alkaline phosphatase; LDH: Lactate dehydrogenase; ESR: Erythrocyte sedimentation rate; CRP: C reactive protein; CEA: Carcinoembryonic antigen; CA 19-9: Carbohydrate antigenic determinant; α FP: α-fetoprotein; NSE: Neuron-specific enolase; Anti HCV: Antibodies for hepatitis C virus; HbsAg: Hepatitis B surface antigen; CgA: Chromogranin A.

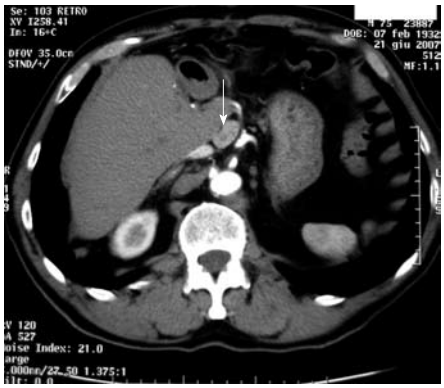

Figure 4 Abdominal CT scan. Results of left hepatectomy show an extended nodular mass measuring about 2.5 cm × 1.5 cm arranged on the back part of the caudate lobe, indicating a lymph node localization of disease.

administered subcutaneously *via* octreotide scan at 2 mo from resection which showed a dishomogeneous distribution of the radioactive drug in the liver site, without any focal images, which warranted further investigation (Figure 3B).

CT scan confirmed the presence of an extended nodular mass measuring about 2.5 cm × 1.5 cm on the back part of the caudate lobe, in close contact with the celiac tripod. The mass was attributable to lymph node recurrence of the disease (Figure 4). The patient showed no signs of evolutive chest disease or any other particular clinical signs.

After a strict clinical and instrumental follow-up period, jointly conducted by an oncologist and a surgeon, the patient's clinical and radiological picture remained stable, as demonstrated by the octreotide therapy. The patient then underwent a surgical lymph node exeresis. The histological finding was compatible with the lymph node metastasis of the neuroendocrine tumor (Figure 5). The postoperative clinical course was uneventful, with a negative follow-up for hematochemical, clinical and radiological investigations at 18 mo post surgery.

DISCUSSION

Neuroendocrine tumors cover a wide range of neoplasms that originate in the neuroendocrine cells that spread throughout the body. Recent studies have suggested an increase in the incidence of these tumors over time[7]. In particular, Maggard *et al*[4] reported a 6.3% increase in 1997 compared with 1973. This could be attributed to an enhanced classification of these tumors and better use of endoscopic techniques for screening purposes. In 1998, 90% of neuroendocrine tumors were reported to occur within the gastrointestinal tract, particularly at the level of the terminal ileum and appendix[8]. However, more recent studies have reported a less frequent gastroenteric involvement (54.%) followed, in decreasing order, by the lung (30.1%), pancreas (2.3%), reproductive system, (1.2%), biliary tract (1.1%), and head and neck (0.4%). As far as the gastrointestinal tract is concerned, a slighter greater involvement of the appendix has been reported compared other sites, such as the small bowel (44.7%) followed by the rectum (19.6%), appendix (16.7%) colon (10.6%) and stomach (7.2%)[4].

PHCTs are rare neuroendocrine tumors, representing 0.3% of all carcinoids, and were first described by Edmonson in 1958[9,10]. Recent studies reported a survey on 95 cases of PHCT[5,11]. The liver is the most frequently involved organ due metastatic disease from extrahepatic neuroendocrine tumors, thus justifying the physician's efforts in ruling out the presence of other diseases before confirming this organ as the primary nature of the tumor[5].

Indeed both the clinical exclusion criteria and the histological confirmation represent a diagnostic means to approach this rare disease.

The clinical onset of neoplasms is often aspecific and related to mass effect on the liver and adjacent organs. Likely symptoms include pain, weight loss, palpable mass, while less common is the classic carcinoid syndrome (skin flushing, abdominal pain and episodes

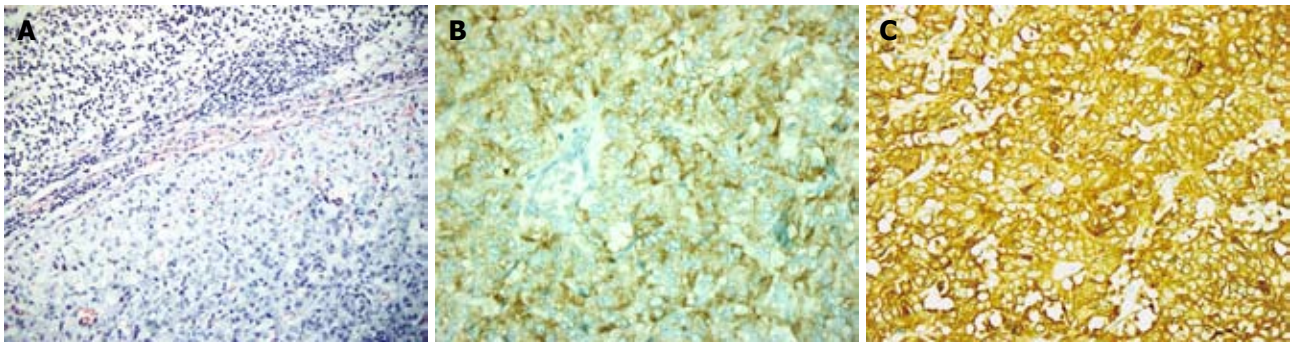

Figure 5 Histological and immunohistochemistry. Proliferation of average sized monomorphic epithelial cells collected in strings and glandular structures (A). Positive immunohistochemistry staining for CgA (B) and NSE (C).

of diarrhea) which are present in 5% of cases[5] and more frequently found in tumors which metastasize to the liver.

The clinical course of PHCT is generally painless compared to other neuroendocrine neoplasms with a more malignant progression. The latter may be characterized by varying degrees of pleomorphism, greater mitotic activity, vascular invasion and necrosis[12]. From the morphological point of view, in accordance with the literature, our patient presented well-differentiated PHCT with low-grade malignancy, minimum pleomorphism, low mitotic index and poor necrotic foci.

The first level diagnosis consisted of non-invasive imaging. A traditional ultrasound scan revealed a hyperecogenic mass containing multiple cystic lesions, while a CT scan confirmed a cystic pattern. Moreover, angiography might demonstrate multiple hypervascular and centrally located radiolucent areas[5]. Classical PET with fluorodeoxyglucose did not prove to be advantageous in neuroendocrine tumor imaging[13]. Thus, a serotonin precursor 11C-5 hydroxy tryptophan was developed as a tracer for PET-scanning, which can be concentrated within carcinoid tumors[13]. Findings using this application are encouraging, allowing the identification of the primary tumor in 84% of cases[14,15]. Primary carcinoid tumors and distant metastasis in patients affected by neuroendocrine tumors are better detected by otreoscan scintigraphy compared to the CT scan and the MRI[16].

The presence of somatostatin receptors within the tumoral cell is best suited for scintigraphy. There are five receptor subtypes (SSTR 1-5) each having different functional properties and binding specificity for the target tissue[16]. Octreotide binds with high affinity to the somatostatin subtype 2 receptor (SSTR 2), which is widely expressed on the cell surface with neuroendocrine characteristics. Certain diagnosis is, however, achieved by fine needle aspiration or biopsy. Immunohistochemistry confirms neuroendocrine origin of PHCT by detecting the markers CgA, NSE, chromostatin, CEA and synaptophysin[17]. Measurement of plasma CgA and repetition of the octreoscan scanning provide the basis for follow-up[5]. Although PHCT appears to be a low malignancy tumor with slow progression, treatment effectiveness and prognosis are difficult to establish owing to its rarity and subsequent lack of prospective data[18]. Surgical resection is the most commonly used therapy and it is considered the treatment of choice[19] in about 85% of primary hepatic carcinoids[18]. This procedure cannot be performed in the 10% of patients affected by metastatic hepatic carcinoid[20,21]. For these cases, as well as for non operable tumors, therapy with radionuclides and the somatostatin analog 177Lu-DOTA-Tyr3-octreotate, are the most modern and promising, not only in terms of stabilization but also with regard to disease regression with minimal toxicity[5,22]. Other therapeutic interventions have been tried for curative and palliative purposes, such as systemic chemotherapy, hepatic artery chemoembolization (only in cases of non resectable or recurrent disease), somatostatin hormone therapy or its analogs performed as a stand-alone therapy or as an adjunct to surgery[5,18]. Hormone therapy is indicated in carcinoids causing functional symptoms, however, no evidence is available as to the control of disease progression. Moreover, this therapy might only exert cytostatic effects[23-25]. Indeed, evidence does exist demonstrating that somatostatin analogs can inhibit tumour growth, at least for a certain period of time[26,27], but further studies are necessary to evaluate this effect.

Recent reports have demonstrated a favourable prognosis at 5 years in 74% of surgically treated cases with an 18% recurrence rate[19]. Post-resection perihepatic lymph node involvement has been infrequently reported in the literature without hepatic involvement, similarly to bone and lung metastasis[18,28].

In 2001, Iwao *et al*[18] analyzed 53 cases of PHCT reported in the English language literature. Accordingly, lymph node involvement occurs in 60% of cases. A case report of 2002[29] confirmed a case of metachrone lymph node metastasis after a 5-year follow-up in one case of surgically treated PHCT.

The case reported here is unique due to its discovery by chance during an abdominal scan which the patient was undergoing for other reasons.

The extremely long evolution, and the absolute lack of pathognomonic symptoms of the disease, resulted in successful diagnosis following a complex process lasting two years. Moreover, the diagnostic course was characterized by the physician's efforts to rule out

extrahepatic neoplasms with possible hepatic metastatic disease.

A diagnostic algorithm proposed by a study published in 2003[23] underlined the need for thorough research into neuroendocrine neoplasms of the small bowel (mid gut), large bowel (hind gut), bronchi (foregut) and pancreas (islet cell). In fact, a small sized lesion can metastasize extensively to liver tissues and might not be detected during a classic diagnostic approach.

In conclusion, a regular clinical and instrumental post surgical review is essential for identifying possible tumor recurrence as well as detecting previously unrecognised primary extrahepatic lesions.

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CASE REPORT

Biliary drainage of the common bile duct with an enteral metal stent

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Abstract

In this case report we present an elderly patient who was referred to our hospital with recurrent episodes of cholangitis that persisted after placement of five metal stents for a distal common bile duct (CBD) stenosis. All metal stents were endoscopically removed from the CBD by forceps after balloon dilatation of the papilla. A profoundly dilated CBD with sludge and concrements was seen. To ensure adequate bile drainage an enteral metal stent was inserted in the CBD. This case shows that proximally migrated uncovered metal stents in the CBD can be safely removed endoscopically under certain circumstances. We suggest that in the case of a CBD drainage problem due to an extremely dilated CBD, placement of an enteral metal stent in the CBD could be considered, especially in patients who are unfit for surgery.

Key words: Cholangitis; Dilated common bile duct; Endoscopic retrograde cholangiopancreatography; Enteral metal stent; Metal stent removal

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INTRODUCTION

Biliary drainage of inoperable malignant bile duct obstruction by a metal stent is preferred over plastic stents due to longer patency[1]. Unfortunately, even metal stents can become dysfunctional[2]. Usually a second stent (metal or plastic) is placed[3] or an attempt to remove the stent endoscopically can be made[4]. We report a case of relapsing cholangitis after placement of 5 metal stents. Removal of the metal stents and insertion of an enteral stent in the common bile duct (CBD) regained adequate drainage.

CASE REPORT

An 84-year old woman was referred to our hospital with relapsing cholangitis since August 2007. Under the suspicion of a malignant distal CBD stricture with stones, a metal stent was placed. As drainage remained inadequate, the patient underwent several endoscopic retrograde cholangiopancreatography (ERCP) and percutaneous transhepatic cholangiography (PTC) procedures with the subsequent placement of 5 uncovered metal 30 French 60 mm Wall stents (Boston Scientific Corporation, Natick, USA) and 2 externally draining PTC drains in the CBD, without clinical improvement.

The patient was referred to our centre in February 2008. Work-up by endoscopy and radiography showed five Wall stents in the CBD (four proximally and one was dislocated distally) and two externally draining 8 French percutaneous drains (Figure 1). To improve drainage the percutaneous drains were replaced by two internal/external 10 French drains. After endoscopic removal of the distally dislocated metal stent with the help of a snare, a large amount of biliary sludge with concrements was drained.

The patient recovered well, but two weeks later, she had to be re-admitted with bile leakage alongside the drains and a poor overall condition due to inadequate drainage (Figure 2).

During ERCP all metal stents were removed. We

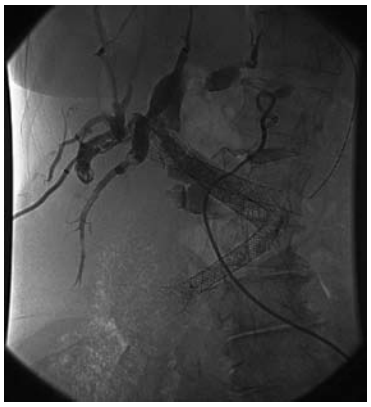

Figure 1 Radiography image. At time of presentation, sludge and stones in the proximal bile ducts with five metal stents in the CBD (four proximally and one distally) and two externally draining 8 French percutaneous drains.

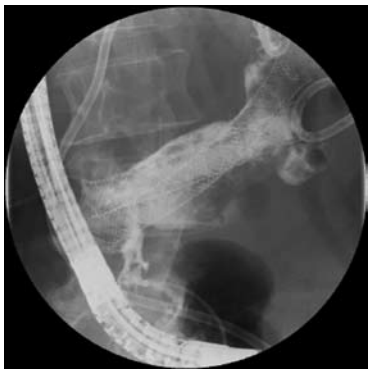

Figure 2 After endoscopic removal of the distal metal stent. The distal CBD was visualized with 4 metal stents proximally in an abnormal position in relation to the CBD.

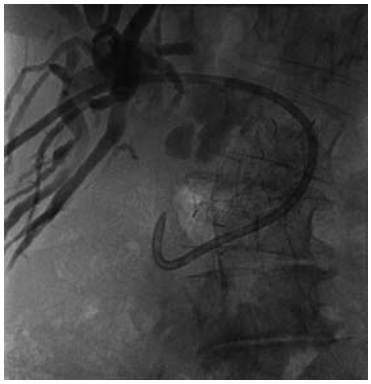

Figure 3 After placement of an enteral metal stent in the dilated CBD.

performed balloon dilatation (18 mm CRE balloon, Boston Scientific International S.A., Nanterre Cedex, France) of the sphincter and the relatively narrow distal CBD to 18 mm. We encountered four metal stents that had been overlapping. The stents were subsequently removed with foreign body forceps. With a balloon and crusher the majority of the concrements and debris was removed. Given the extreme dilatation of the CBD, up to 5 cm in diameter, with a profound angulation of the distal CBD and in retrospect no malignant stricture, an enteral metal stent was placed (WallFlex enteral duodenal stent 22 mm × 60 mm, Boston Scientific Corporation, Natic, USA) to prevent further obstruction by kinking[5] (Figure 3). Two internal/external drains were temporarily placed through the "enteral" stent to flush the bile ducts to remove the remaining debris.

Six months later our patient is still without signs of cholangitis.

DISCUSSION

In this case the following aspects were identified: After several interventions, the CBD drainage was still inadequate and the general condition of the patient excluded surgery as an option. To improve drainage, we removed all metal stents. The endoscopic removal of distal dislocated metal stents by forceps or a loop was previously described by Matsushita *et al*[4]. However, in our case we removed, in addition to the distally dislocated stent, a total of 4 metal stents that had migrated proximally in the CBD. To our knowledge, this has never been described. Usually these proximally migrated stents are fixed into their surroundings due to the radial expending force and ingrowth of bile duct epithelium. Secondly, these stents were located beyond the papilla which had to be dilated in order to reach the stents. Normally in the case of stent dysfunction, plastic stents or a second metal stent is introduced through the obstructing metal stent. However, the metal stents seemed to attribute to the increased angulation of the distal CBD and therefore had to be removed. In this particular case, safe removal was possible because the metal stents were free floating within an extremely dilated CBD. This left the patient with a widened CBD with a secondary distal angulation contributing to recurrent obstruction. Normally a hepaticojejunostomy would solve these problems, however, her general condition combined with her age forced us to look for other options. We chose to place a large diameter enteral metal stent in the distal CBD to avoid dislocation and kinking of the widened CBD. This procedure was successfully used by Diehl *et al*[5] to stent a wide CBD due to a choledochal cyst. In our patient we achieved adequate drainage, the stent remained in position and no recurrence of cholangitis has occurred for more than 6 mo.

Proximally migrated uncovered metal stents in the CBD can be safely removed endoscopically under certain circumstances. We suggest that in the case of a CBD drainage problem due to an extremely dilated CBD, placement of an enteral metal stent in the CBD could be considered, especially in patients who are unfit for surgery.

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CASE REPORT

Solitary extramedullary plasmacytoma in retroperitoneum: A case report and review of the literature

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Abstract

Extramedullary plasmacytoma (EPM) is a plasma cell tumor arising outside of the bone marrow. Solitary EMP is an uncommon neoplasm and rarely occurs in the retroperitoneum and lacks distinctive clinical manifestations. We report a 26-year-old man with a solitary EMP in the retroperitoneum and discuss its clinical features, diagnosis and treatment.

Key words: Extramedullary plasmacytoma; Retroperitoneal neoplasm; Computed tomography; Histopathology

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INTRODUCTION

Extramedullary plasmacytoma (EMP), accounting for approximately 3% of all plasma cell neoplasms, results from uncontrolled plasma cell proliferation and consists of monoclonal plasmacytic infiltration without bone marrow involvement[1]. Approximately 80%-90% of EMPs involve mucosa-associated lymphoid tissue of the upper airway and 75% of them involve the nasal and paranasal regions, while retroperitoneal infiltration is very rare[2]. We report a 26-year-old man with a solitary EMP in the retroperitoneum.

CASE REPORT

A 26-year-old man was referred to our hospital with a history of abdominal distention and effort intolerance persisting for the previous 2 mo. He had no history of fever, weight loss, bladder or bowel dysfunction, and back pain. Physical examination revealed an irregular, firm, non-tender mass occupying almost the whole abdomen. He had no icterus or lymphadenopathy with normal tests. An abdominal computed tomography (CT) scanning showed a large heterogeneous mass in the right retroperitoneal region, surrounding the posterior portion of the right kidney and compressing the right kidney (Figure 1A). The tumor tissue was slightly enhanced after injection of a contrast medium (Figure 1B).

Laboratory test revealed 2.8 × 10^9/L white blood cells (WBC) (normal range 4-10 × 10^9/L), 119 g/L hemoglobin (Hb) (normal range 120-160 g/L), 122 × 10^9/L platelets (PLT) (normal range 100-300 × 10^9/L), and normal serum levels of creatinine, blood urea nitrogen, amylase, hepatic enzymes, electrolytes including calcium and phosphorus. No erythrocyte or protein was observed in his urine.

Fine needle aspiration cytology of the mass was not done because of refusal of his parents who were afraid of needle track implantation. Thereafter, an extensive resection of the tumor including extirpation of the right kidney, and part of the liver was performed. Postoperative recovery was uneventful.

Histopathology revealed a 30 cm × 16 cm × 10 cm tumor surrounding the posterior portion of the right kidney with adherent liver. Microscopy showed diffusive infiltration of polygonal cells in the retroperitoneum.

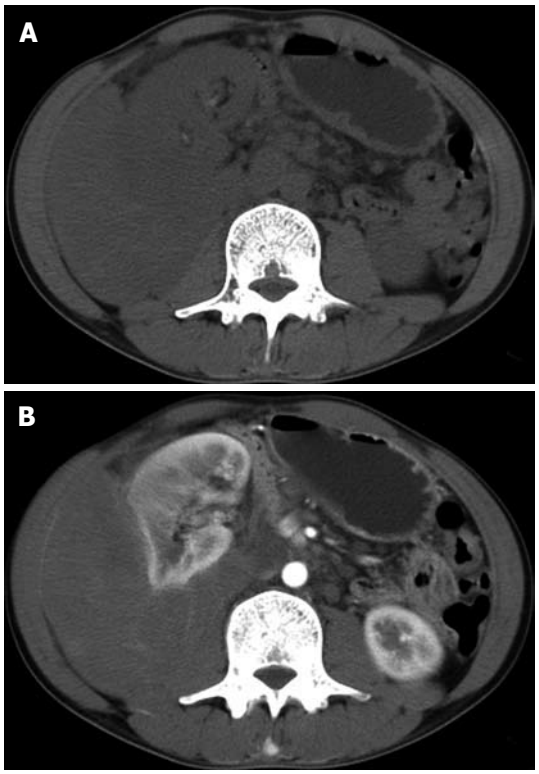

Figure 1 Abdominal computed tomography showing a large heterogeneous mass in the right retroperitoneal region, surrounding the posterior portion of the right kidney and compressing the right kidney (A), and slightly enhanced tumor tissue after injection of a contrast medium (B).

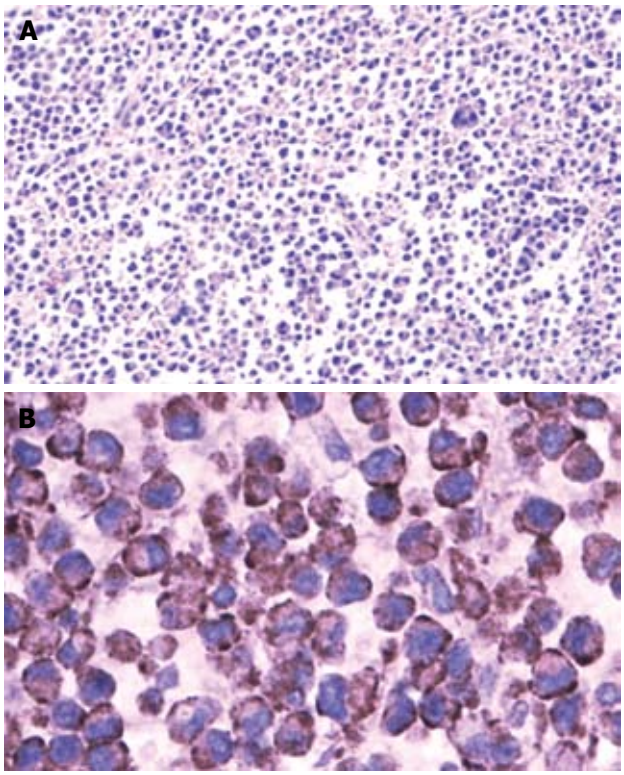

Figure 2 Tumor cells with homogenous amphophilic cytoplasm, whell-type and asymmetric nuclei, coarsely stippled chromatin, some acidophilic nucleoli, and occasional binucleate (A) (HE, × 100) and positive tumor cells for CD138 (B) (× 400) under microscope.

Homogeneous amphophilic cytoplasm, wheel-type and asymmetric nuclei, coarsely stippled chromatin, and some acidophilic nucleoli were observed in the tumor cells. Binucleate cells were also occasionally observed (Figure 2A). Immunohistochemistry demonstrated tumor cells were positive for CD138 (Figure 2B), Bcl-2 and VS38C, but negative for CD20, CD3, CD79a, CK, CD38, S-100, CD5, CK, myosin and CD10.

To confirm the diagnosis of EMP, further investigations were done after surgery. Serum IgG was 25.4 g/L (normal range 6.94-16.20 g/L). Serum IgA and IgM levels were within normal range. No Bence-Jones protein was detected in his urine. Iliac crest bone marrow aspiration and biopsy did not find any plasmacytic infiltration. A skeletal survey revealed no osteolytic lesions.

DISCUSSION

Solitary bone and extramedullary plasmacytomas are rare plasma cell proliferative disorders. Their diagnosis is based on the monoclonal plasma cell infiltration at a single disease site and the exclusion of systemic myeloma[3]. WBC and hemoglobin were slightly abnormal, and iliac crest bone marrow aspiration and biopsy showed no plasmacytic infiltration in our case. We ascribed these abnormalities to the fact that he worked as a painter for 8 years prior to surgery. Hematopathy can be found in workers exposed to benzene[4].

EMP occurs most commonly in the head and neck region, followed by gastrointestinal (GI) tract, central nervous system (CNS), thyroid, breast, parotid gland, testis, and lymph nodes[5].

Solitary EMP rarely occurs in the retroperitoneum. Cases of retroperitoneal EMP have different clinical manifestations, such as renal failure due to bilateral renal vein occlusion[6], flank pain, hematuria due to thrombosis of the renal vein[7], obstructive jaundice[8], abdominal distention and pain[9,10], and hyperamylasemia[11]. However, our patient presented with only abdominal distention and effort intolerance.

Retroperitoneal EMP should be differentially diagnosed from lymphoplasmacytic lymphoma and immunoblastic lymphoma[12]. Immunohistochemistry is used for its final diagnosis. In our case, microscopy showed that the tumor cells might be origined from plasmacytic cells confirmed by immunohistochemistry.

Preoperative CT scanning does not contribute to its differential diagnosis from other tumors, while preoperative angiography can indicate the vessels feeding the mass and the correlation to other vessels. Serum electrophoresis can help its diagnosis by finding the M band. However, we considered the mass as a common type of tumors, such as schwannoma, sarcoma before operation and serum electrophoresis was not done.

No clear guidelines for treatment of EMP are available due to its rarity and variable presentations. EMP is highly radiosensitive with excellent results (< 10% of local recurrences and about 50%-65% of patients remain free of disease for > 10 years)[13]. However, it is associated with a high morbidity particularly when

used for large retroperitoneal tumors. It was reported that there is no evidence that retroperitoneal EMP progresses one year after chemotherapy in combination with radiotherapy[14]. Chen *et al*[8] have reported a case of retroperitoneal EMP accompanying obstructive jaundice, who showed a complete response to sequential radiotherapy and chemotherapy.

Sharma *et al*[10] performed a complete surgical resection of a large bulky retroperitoneal EMP when the patient did not respond to chemotherapy, and found that the patient was symptom free 16 mo post surgery. Our patient did not receive chemotherapy or radiotherapy prior to operation. He was under observation 2 mo after surgery and remained asymptomatic when we wrote this paper.

In summary, EMP should be considered whenever a retroperitoneal soft tissue mass is identified.

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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

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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
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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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
London, UK
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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Instructions to authors

GENERAL INFORMATION

World Journal of Gastroenterology (*World J Gastroenterol* ISSN 1007-9327 CN 14-1219/R) is a weekly open-access (OA) peer-reviewed journal supported by an editorial board consisting of 1179 experts in gastroenterology and hepatology from 60 countries.

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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insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

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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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[2]Passed away on June 11, 2007
[3]Passed away on June 14, 2008

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EDITORIAL

Non-invasive biomarkers for monitoring the fibrogenic process in liver: A short survey

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Abstract

The clinical course of chronic liver diseases is significantly dependent on the progression rate and the extent of fibrosis, i.e. the non-structured replacement of necrotic parenchyma by extracellular matrix. Fibrogenesis, i.e. the development of fibrosis can be regarded as an unlimited wound healing process, which is based on matrix (connective tissue) synthesis in activated hepatic stellate cells, fibroblasts (fibrocytes), hepatocytes and biliary epithelial cells, which are converted to matrix-producing (myo-)fibroblasts by a process defined as epithelial-mesenchymal transition. Blood (non-invasive) biomarkers of fibrogenesis and fibrosis can be divided into class Ⅰ and class Ⅱ analytes. Class Ⅰ biomarkers are those single tests, which are based on the pathophysiology of fibrosis, whereas class Ⅱ biomarkers are mostly multiparametric algorithms, which have been statistically evaluated with regard to the detection and activity of ongoing fibrosis. Currently available markers fulfil the criteria of ideal clinical-chemical tests only partially, but increased understanding of the complex pathogenesis of fibrosis offers additional ways for pathophysiologically well based serum (plasma) biomarkers. They include TGF-β-driven marker proteins, bone marrow-derived cells (fibrocytes), and cytokines, which govern pro- and anti-fibrotic activities. Proteomic and glycomic approaches of serum are under investigation to set up specific protein or carbohydrate profiles in patients with liver fibrosis. These and other novel parameters will supplement or eventually replace liver biopsy/histology, high resolution imaging analysis, and elastography for the detection and monitoring of patients at risk of developing liver fibrosis.

Key words: Biochemical markers; Diagnostic validity; Liver fibrosis; Monitoring; Multiparametric algorithms; Non-invasive diagnostic tools

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INTRODUCTION

Tissue fibrosis is characterized by the excess deposition of extracellular matrix (ECM) involving molecular and histological re-arrangement of various types of collagens, proteoglycans, structural glycoproteins and hyaluronan (Figure 1). It is a hallmark of liver cirrhosis and contributes significantly to the deleterious outcome of chronic liver diseases[1]. The deposition of ECM in the space of Disse (perisinusoidal fibrosis) between the sinusoidal surface of hepatocytes and the endothelial cell layer of liver sinusoids, the generation of (incomplete) subendothelial basement membranes, and the strangulation of hepatocytes by surrounding matrix impair not only the blood flow through the organ, but also the biosynthetic function of hepatocytes and the clearance capability of these and other cell types[2].

The molecular pathogenesis of the fibrotic transition of liver parenchyma turns out to be a muti-faceted process largely due to the activation of resting, vitamin A-storing stellate cells to matrix-producing myofibroblasts[3,4] in the immediate neighbourhood of hepatocytes, to the phenotypic switch of hepatocytes and bile duct epithelial

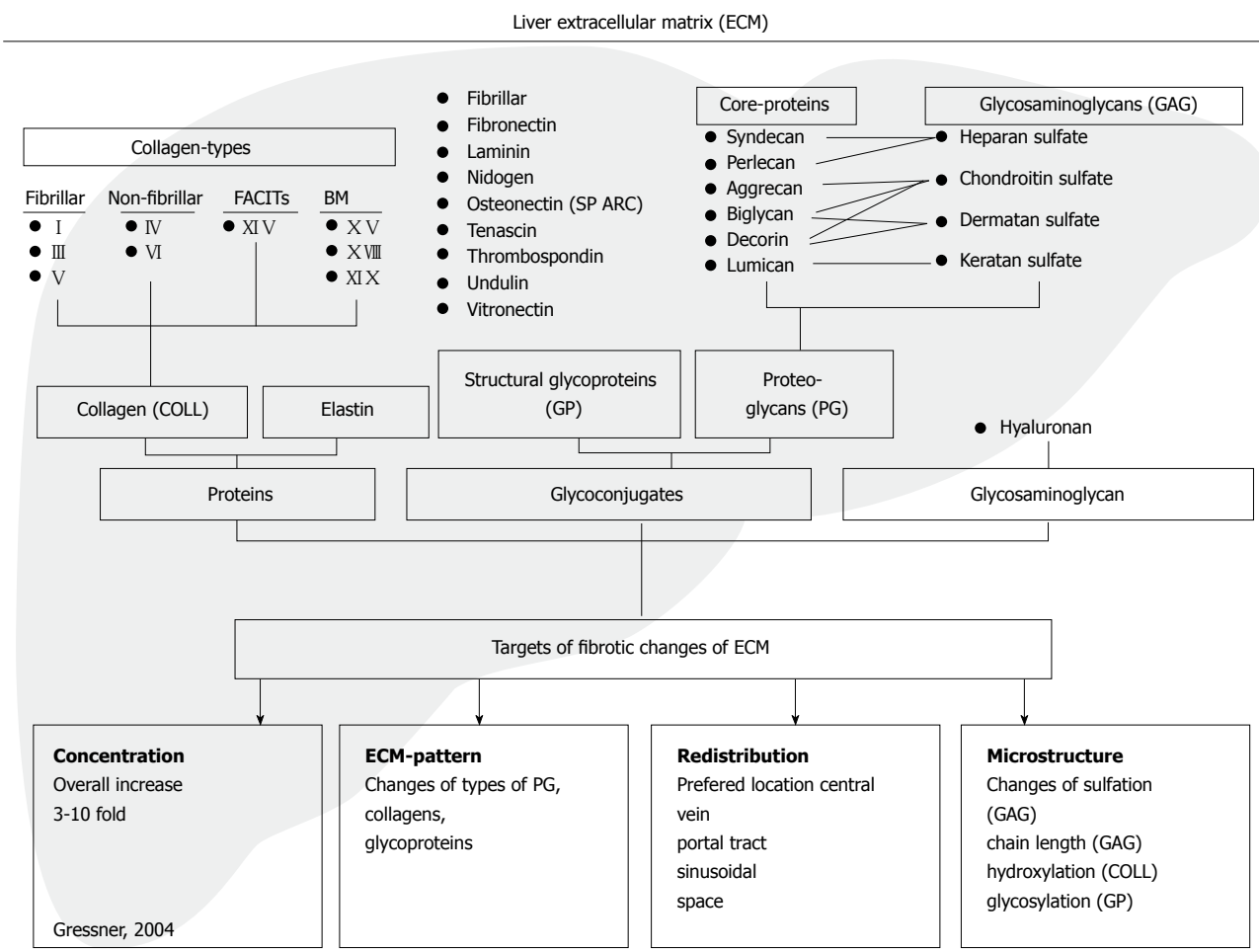

Figure 1 Components of the extracellular matrix (connective tissue) of the fibrotic liver and their major changes. The binding of glycosaminoglycans (GAG) to the respective core proteins (CP) of proteoglycans (PG) are shown. BM: Basement membranes; FACIT: Fibril-associated collagens with interrupted triple-helices.

cells to fibroblasts termed epithelial-mesenchymal transition (EMT)[5-7], and to the influx of bone marrow-derived cells (fibrocytes) reaching the liver via the systemic circulation[8,9] (Figure 2). The fractional contribution of these pathways to fibrosis depends on the underlying disease and probably on the stage of the fibrotic transition[2]. The activation of stellate cells results from interaction with damaged hepatocytes, activated Kupffer cells, disintegrated platelets and various subfractions of leucocytes. Among the cytokines involved in the pathogenetic processes, TGF-β plays a dominant role, but PDGF, endothelin-1, VEGF, and others also contribute significantly. Antagonistic (antifibrotic) mediators might also exist among which bone morphogenetic protein (BMP)-7 plays an important role, e.g. in the inhibition of EMT-derived fibroblasts[10].

The pathogenetic complexity is mirrored by multiple approaches of a clinical diagnosis and a follow-up of ongoing liver fibrosis.

The widely used diagnostic "gold standard" of liver biopsy has many draw-backs besides its invasiveness such as sampling error (around 1/50 000th of liver mass is obtained), irreproducible sample quality depending on length and size of the tissue specimen (coefficient of variation 45%-35%) and a histological evaluation strictly dependent on the experience of the pathologist (observer error)[11]. Therefore, the development of non-invasive, objective and quantitative serum- or plasma-based biomarkers of fibrogenesis is an important goal, which can be approached by the assessment of two, principally different lines of blood-borne (non-invasive) analytes: Class Ⅰ and class Ⅱ serum fibrosis markers.

CLASSIFICATION OF CIRCULATING BIOMARKERS OF FIBROSIS

Class Ⅰ fibrosis biomarkers are pathophysiologically derived from ECM turnover and/or from changes of the fibrogenic cell types, in particular hepatic stellate cells (HSC) and (myo-)fibroblasts[3]. They should reflect the activity of the fibrogenic and/or fibrolytic process and, thus, remodelling of ECM. These biomarkers do not indicate the extent of connective tissue deposition, i.e. the stage of fibrotic transition of the organ. Frequently, they involve costly laboratory tests and are the result of translation of fibrogenic mechanisms into clinical application. Thus, their selection is hypothesis-driven.

Class Ⅱ fibrosis biomarkers mostly estimate the degree of fibrosis (extent of ECM deposition). In general, they comprise common clinical-chemical tests (enzymes, proteins, coagulation factors), which do not necessarily

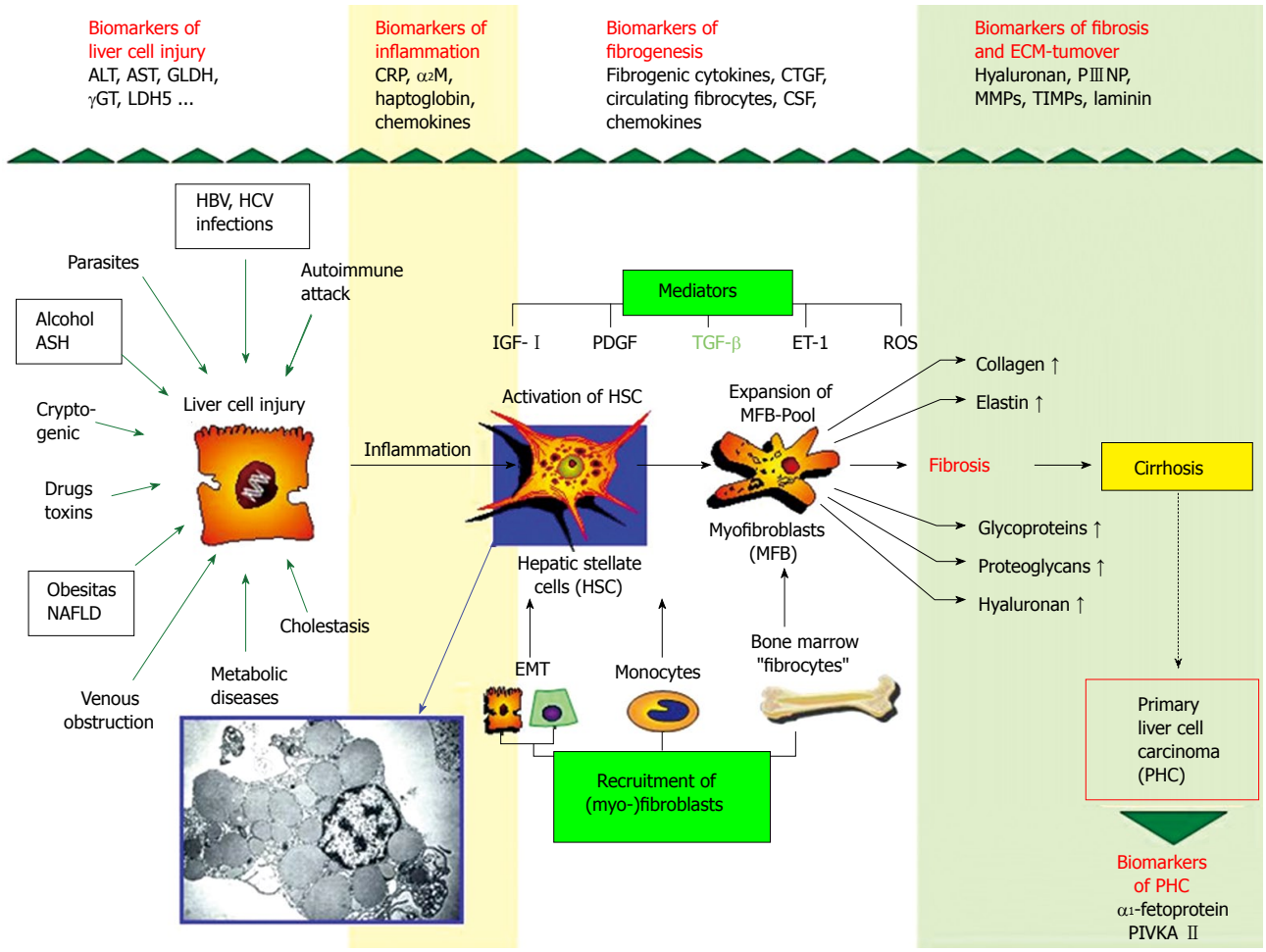

Figure 2 Synopsis of pathogenetic mechanisms of liver fibrosis (fibrogenesis). The cells produce an increase in extracellular matrix derived from activated hepatic stellate cells (HSC)/expended pool of myofibroblasts (MFB) produce various components of the extracellular matrix (fibrosis) leading to cirrhosis. Newly recognized pathogenetic mechanisms point to the (i) influx of bone marrow-derived cells (fibrocytes) to the liver, (ii) to circulating monocytes and to their TGF-β-driven differentiation to fibroblasts and (iii) to the epithelial-mesenchymal transition (EMT) of hepatocytes and bile duct epithelial cells to fibroblasts. All three complementary mechanisms enlarge the pool of matrix-synthesizing (myo-)fibroblasts. Some important fibrogenic mediators are transforming growth factor (TGF)-β, platelet-derived growth factor (PDGF), insulin-like growth factor I (IGF- I), endothelin-1 (ET-1), and reactive oxygen metabolites (ROS). Abbreviations: ASH: Alcoholic steatohepatitis; NAFLD: Non-alcoholic fatty liver disease. The insert shows an electron micrograph of hepatic stellate cells containing numerous lipid droplets.

reflect ECM metabolism or fibrogenic cell changes. Their pathobiochemical relationship with fibrogenesis is indirect if at all. Thus, their selection is not hypothesis-driven, but empiric. The markers are standard laboratory tests and are integrated into multiparametric panels.

In general, both types of serum biomarkers follow different pathophysiological concepts. Class I markers inform about "what is going on" (grade of fibrogenic activity), class II markers indicate "where fibrosis is" (stage of fibrosis).

Class I fibrosis biomarkers

These biomarkers are components of the connective tissue (matrix) increasingly expressed by activated hepatic stellate cells (HSC) and (myo-)fibroblasts[12], have a delayed clearance by Kupffer cells or sinusoidal endothelial cells in the liver due to metabolic dysfunction and/or hemodynamic bypasses, or are increasingly expressed mediators of fibrogenesis such as TGF-β. Taken together, of the several procollagen and collagen fragments proposed, only the N-terminal propeptide of type III procollagen (PIIINP) has reached a limited clinical application, but not widespread acceptance[13]. Sensitivities of about 76%-78% and specificities of 71%-81% have been reported, which can be increased up to 88%, if combined with additional collagen fragment markers. It should be emphasized that PIIINP is not a liver-specific biomarker. Similarly, structural glycoproteins (e.g. undulin, tenascin), biosynthetic (e.g. prolyl hydroxylase) or catabolic enzymes (e.g. matrix metalloproteinases) of collagen and other ECM-components have not been convincing in the detection, grading, and staging of fibrosis (Table 1). Several studies have shown that hyaluronic acid (hyaluronan) is currently the best class I biomarker of fibrosis having an area under the receiver operating characteristics (AUROC) of 0.97, a sensitivity of 86%-100%, and a specificity of about 88% in a recent investigation of cirrhosis due to non-alcoholic fatty liver disease[14] and other aetiologies. Since the negative predictive value of hyaluronan at a cut off value of 60 μg/L is much higher (98%-100%) than the positive predictive value (61%), the main utility of serum hyaluronan lies in its ability to exclude advanced fibrosis and cirrhosis. Its

Table 1 Class I biomarkers of liver fibrogenesis

	Specimen			Method
	Serum	Urine	Liver biopsy	
Extracellular matrix-related enzymes				
Enzyme				
Prolyl hydroxylase	+	-	+	Radioenzymatic, RIA
Monoamine-oxidase	+	-	(+)	Enzymatic
Lysyl oxidase	+	-	+	RIA
Lysyl hydroxylase	+	-	-	RIA
Galactosylhydroxylysyl-glucosyltransferase	+	-	+	RIA
Collagen peptidase	+	-	+	Enzymatic
N-Acetyl-β-D-glucosaminidase	+	+	+	Enzymatic
Collagen fragments and split products				
Type of collagen				
Type Ⅰ-procollagen				
N-terminal propeptide (PINP)	+	-	+	ELISA
C-terminal propeptide (PICP)	+	-	+	RIA
Type Ⅲ-Procollagen				
Intact Procollagen	+	-	-	RIA
N-terminal propeptide (PⅢNP)				
Complete propeptide (Col 1-3)	+	-	-	RIA
Globular domain of Propeptide (Col-1)	+	-	-	RIA
Type Ⅳ-Collagen				
NC1-fragment (C-terminal)				
crosslinking domain (PIVP)	+	+	-	ELISA, RIA
7S domain ("7S Collagen")	+	+	-	RIA
Type Ⅵ-Collagen	+	+	+	RIA
Glycoproteins and matrix-metalloproteinase (inhibitors)				
Marker				
Laminin, P1-fragment	+	-	-	RIA, EIA
Undulin	+	-	-	EIA
Vitronectin	+	-	-	EIA
Tenascin	+	-	-	ELISA
YKL-40	+	-	+	RIA/ELISA
(pro)matrix metalloproteinase (MMP-2)	+	-	-	ELISA
Tissue inhibitor of metalloproteinases (TIMP-1, TIMP-2)	+	-	-	ELISA
sICAM-1 (soluble intercellular adhesion molecule, sCD54)				
sVCAM-1 (soluble vascular cell adhesion molecule, sCD106)	+	-	-	ELISA
Glycosaminoglycans				
Marker				
Hyaluronic acid (Hyaluronan)	+	-	-	Radioligand assay ELISA
Molecular mediators				
Marker				
Transforming growth factor β (TGF-β)	+	-	+	ELISA
Connective tissue growth factor (CTGF/CCN2)	+	?	+	ELISA

stimulated synthesis in activated HSC, secretion into the sinusoidal blood stream, and short half life of 2-9 min in the circulation are good suppositions for a valid fibrosis biomarker. Laminin was reported to be a predictor of portal hypertension since significantly elevated concentrations were found under these conditions[15]. TGF-β concentration in plasma is elevated in and correlates with the severity of liver disease and is suggested to be a non-invasive biomarker of fibrosis. However, the significant correlation with AST and ALT activity[16] and the pathobiochemical finding that substantial amounts of TGF-β are localized in hepatocytes and released into the medium if hepatocytes are permeabilized[17] suggest that the elevation of TGF-β is a marker of necrosis instead of fibrogenesis.

Preliminary studies point to connective tissue growth factor (CTGF/CCN2) in serum as an innovative class Ⅰ biomarker of fibrogenesis[18]. This 38 kDa protein is synthesized not only in HSC, but also in hepatocytes where the expression and secretion is strongly dependent on TGF-β[19,20]. Accordingly, the expression of the TGF-β down-stream mediator CTGF in fibrotic liver tissue is up-regulated and its concentration in blood is elevated if fibrogenesis is occurring. There is a correlation between CTGF levels and fibrogenesis, because the levels decrease in fully developed, end-stage cirrhosis, compared to fibrosis. The AUCs for fibrosis *vs* control and cirrhosis *vs* control were calculated to be 0.955 and 0.887, respectively, the sensitivities 100% and 84%, respectively, the specificities 89% and 85%, respectively[18]. These criteria suggest that CTGF is a potentially valuable class Ⅰ biomarker of active fibrogenesis.

Recently, the glycoprotein YKL-40 ("chondrex", molecular mass 40 kDa), which is likely a growth factor for fibroblasts and endothelial cells, was shown to be

Table 2 Class Ⅱ biomarkers of liver fibrogenesis

Index	Parameters	Chronic liver disease	Sensitivity (%)	Specificity (%)
PGAA-Index	Prothrombin time, γGT, apolipoprotein A1, α2-macroglobulin	Alcohol	79	89
Bonacini-Index	ALT/AST-ratio, INR, platelet count	HCV	46	98
Sheth-Index	AST/ALT (De Ritis)	HCV	53	100
Park-Index		HCV	47	96
PGA-Index	Prothrombin time, γGT, apolipoprotein A1	Mixed	91	81
Fortunato-Score	Fibronectin, prothrombin time, PCHE, ALT, Mn-SOD, β-NAG	HCV		94
Fibrotest (Fibro-Score)	Haptoglobin, α2-macroglobulin, apolipoprotein A1, γGT, bilirubin	HCV HBV	75	85
Pohl-Score	AST/ALT-ratio, platelet count	HCV	41	99
Actitest	Fibrotest + ALT	HCV		
Forns-Index	Age, platelet count, γGT, cholesterol	HCV	94	51
Wai-Index (APRI)	AST, platelet count	HCV	89	75
Rosenberg-Score (ELF-Score)	PⅢNP, hyaluronan, TIMP-1	Mixed	90	41
Patel-Index (FibroSpect)	Hyaluronan, TIMP-1, α2-macroglobulin	HCV	77	73
Sud-Index (fibrosis probability-index, FPI)	Age, AST, cholesterol, insulin resistance (HOMA), past alcohol intake	HCV	96	44
Leroy-Score	PⅢNP, MMP-1	HCV	60	92
Fibrometer test	Platelet count, prothrombin index, AST, α2-macro-globulin, hyaluronan, urea, age	Mixed	81	84
Hepascore	Bilirubin, γGT, hyaluronan, α2-macroglobulin, age, gender	HCV	63	89
Testa-Index	Platelet count/spleen diameter-ratio	HCV	78	79
FIB-4	Platelet count, AST, ALT, age	HCV/HIV	70	74
FibroIndex	Platelet count, AST, γ-globulin	HCV	38	97

GGT: γ-glutamyltransferase; PⅢNP: N-terminal propeptide of type Ⅲ procollagen; TIMP: Tissue inhibitors of metalloproteinases; MMP: Matrix metalloproteinases; β-NAG: N-acetyl-β-glucosaminidase; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; INR: International normalized ratio.

strongly expressed in human liver tissue[21]. In particular, HSC contain YKL-40 mRNA. Several studies have found elevated YKL-40 concentrations in sera of patients with liver diseases. A sensitivity and specificity of around 80% and an AUC of 0.81 for fibrosis have been reported for HCV-patients[22], for those with alcoholic liver disease, a specificity of 88% and a low sensitivity of 51% were calculated[23]. Serum concentrations of this protein correlated with other ECM products secreted by HSC and fibroblasts, e.g. PⅢNP, hyaluronan, MMP-2, and TIMP-1. It is claimed that YKL-40 concentrations reflect the degree of liver fibrosis but extensive clinical evaluation is still required and other inflammatory diseases as potential causes of YKL-40 elevations have to be excluded. In addition, the expression of this protein is not restricted to the liver, but occurs in chondrocytes (synovial fluid), bone cells, vascular smooth muscle cells and therefore non-specific to the liver[21].

Class Ⅱ fibrosis biomarkers

This category comprises a rapidly increasing, wide variety of biochemical scores and multiparameter combinations (biomarker panels), which are selected by various statistical models and mathematical algorithms, e.g. multiple logistic regression analysis. They fulfil the most appropriate diagnostic criteria for the detection and staging of fibrosis and to a lesser extent for grading of fibrogenesis. In general, the panels consist of rather simple (standard) laboratory tests, which are subject to changes in the serum or plasma of fibrotic and cirrhotic patients (Table 2). Several of the parameters included in the more than 20 scores currently available have no pathophysiological relation to fibrogenesis. Some of them have an indirect relation, and only a few parameters can be regarded as being directly related to fibrogenesis. The parameters measured comprise those of necrosis such as ALT and AST, coagulation-dependent tests, transport proteins, bilirubin and some ECM-parameters. Frequently, the reduction of platelet counts in cirrhotic patients is included. Most prevalent are the Fibrotest™ and for necro-inflammatory activity the Actitest™ (Biopredictive, Paris, France)[24]. These tests are based on γ-glutamyl-transferase (γ-GT), total bilirubin, haptoglobin, α2-macroglobulin, apolipoprotein A1, and for the Actitest additionally on alanine-aminotransferase (ALT)[25]. The data of Fibrotest and Actitest are calculated with a patented artificial intelligence algorithm to give measures of fibrosis stage and necro-inflammatory grade (activity), respectively. The Wai-score based on aspartate-aminotransferase (AST), alkaline phosphatase and platelet count[26], the ELF-test based on TIMP-1, PⅢNP, hyaluronan[27], and the Hepascore based on bilirubin, γ-GT, hyaluronan, α2-macroglobulin, age and gender[28] are further scores with up to now limited clinical application. The Fibrotest was shown to be a better predictor than biopsy staging for HCV complications and death[25]. Recently, Fibrotest™ and Actitest™ were included to detect biomarkers for the prediction of liver steatosis (Steato-test™), alcoholic steato-hepatitis (ASH-test™), and non-alcoholic steato-hepatitis (NASH-test™) by supplementation with serum cholesterol, triglycerides, glucose (and AST for NASH-test) adjusted for age,

Table 3 Future candidate biomarkers of non-invasive diagnosis and follow-up of liver fibrogenesis

Biomarker	Specimen	Assay technology	Pathobiochemical basis
CTGF	Serum	Immunoassay	TGF-β induced expression in and secretion by hepatocytes and hepatic stellate cells
Fibrocytes	Blood, buffy coat	Flow cytometry of $CD34^+$, $CD45^+$, Coll I^+ cells qPCR	Supplementation of local fibroblasts at site of liver injury by bone-marrow derived fibrocytes
BMP-7	Serum	Immunoassay	Antagonist of TGF-β, inhibitor of EMT
G-CSF GM-CSF M-CSF	Blood	Immunoassays	Mobilization of bone-marrow derived fibrocytes
Proteomics	Serum	Mass spectrometry (MS)	Fibrosis-specific serum protein profiles
Glycomics	Serum	Adaptation of DNA-sequencer/fragment analyzer technology to profiling of desialylated N-linked oligo-saccharides	Fibrosis-specific profiles of desialylated serum protein linked oligosaccharides (N-glycans)
Xylosyl-transferase (EC 2.4.2.26)	Serum	LC-MS/MS	Key enzyme in the biosynthesis of glycosaminoglycan chains in proteoglycans, e.g. in hepatic stellate cells and hepatocytes

gender, and body mass index (BMI)[29]. The diagnostic criteria elaborated in a large cohort of patients suggested that the Steato-test was a simple and non-invasive quantitative measure of liver steatosis and the NASH-test was a useful screening procedure for advanced fibrosis and NASH in patients with various metabolic syndromes[29]. FibroMax™ (Biopredictive) was recently developed as a method of combining calculations of these fibrosis-related tests in a single procedure. Comparative evaluation of class Ⅱ serum biomarker panels, however, did not highlight their clinical superiority if liver biopsy was used as the reference method[30]. Since only about 40% of the results were assigned to be correct, a fraction of about 50%-70% was inaccurate with regard to the staging of fibrosis severity and a small fraction of results was even incorrect[30]. Thus, currently suggested multi-parameter approaches with class Ⅱ fibrosis biomarker panels have to be used with caution in clinical practice. A successful approach to improve the diagnostic accuracy of the panel markers in chronic hepatitis C might be their stepwise combination[31]. By combining the sequential algorithms of APRI, Forns' index and Fibrotest (Table 2) the diagnostic performance could be significantly improved resulting in a 50%-70% reduction in the need for liver biopsy[31]. Recently, a comparison of the diagnostic power of up to five class Ⅱ biomarkers led to suggestions to strongly increase their overall accuracy which would, thus, reduce the need for a liver biopsy from 56% to 0% in chronic hepatitis C[32]. Additionally, an algorithm based on the AST-to-platelet-ratio-index (APRI) and liver surface ultrasound nodularity showed a strong diagnostic power making liver biopsy unnecessary[33].

It should be emphasized that the combination of individually assessed parameters necessarily creates a relatively high variance due to the imprecision of each separate measurement[34]. Coefficients of variation range from series to series and are usually between 3% and 6% for common clinical-chemical parameters and from 4% to more than 12% for hyaluronan, PⅢNP, and other matrix parameters. Furthermore, and even more important is the lack of standardized assays for many of these parameters, which excludes the general use of cut-offs and algorithms[34].

DEVELOPMENTS OF INNOVATIVE BIOMARKERS

A growing understanding of the pathogenesis of hepatic fibrosis has indicated potentially powerful non-invasive (blood) biomarkers of hepatic fibrogenesis and fibrosis (Table 3). CTGF/CCN2 was already mentioned as a pluripotent downstream modulator of TGF-β, and was found to be up-regulated by TGF-β in hepatocytes. Although most CTGF will only have a defined paracrine function in fibrogenic tissue, a certain fraction spills over into the circulation, resulting in elevated serum concentrations during active fibrogenesis[18]. The circulating level of CTGF might be an objective and sensitive measure of ongoing fibrogenesis in necro-inflammatory liver tissue.

Bone-marrow-derived fibrocytes might offer new approaches not only for understanding the pathogenesis, but also for the diagnosis of liver fibrosis. Fibrocytes are circulating progenitor cells (CD34 positive) of hematopoietic origin (CD45 positive) capable of differentiating into diverse mesenchymal cell types[35]. The additional markers of fibrocytes, i.e. positivity of type Ⅰ collagen and the CXCR4 chemokine expression can be used to quantitate this special sub-population of circulating leucocytes applying quantitative PCR and/or flow cytometry. The determination of the colony stimulating factors M-CSF, G-CSF, and GM-CSF, which are increasingly expressed in fibrotic liver tissue and elevated in serum[36], are possibly involved in the mobilisation of fibrocytes from the bone marrow and their homing in the liver during fibrogenesis. These factors may be further candidates for diagnostic evaluation.

A new, but currently still controversial aspect of fibrogenesis is epithelial-mesenchymal transition (EMT) of hepatocytes and biliary epithelial cells, respectively, to (myo-)fibroblasts[2]. EMT is governed by the balance of TGF-β (pro-EMT) and its antagonist, i.e. BMP-7 (anti-EMT). In addition to its anti-EMT effect, BMP-7 was shown to have anti-apoptotic and anti-inflammatory activities. Thus, the measurement of BMP-7 alone or even in relation to TGF-β in serum might reflect the activity of fibrogenesis and, hence, the velocity of

fibrotic organ transition[37].

Xylosyltransferase (XT), a key enzyme in the biosynthesis of glycosaminoglycans in proteoglycans, was shown to have increased activities in the serum of patients with connective tissue diseases. With HPLC-tandem mass spectrometry, measurements in large cohorts of liver fibrotic patients may to be possible[38]. Since HSC in fibrotic liver tissue (myofibroblasts) have a greatly stimulated proteoglycan synthesis[39], XT activity in serum might be a promising class I biomarker of fibrogenesis.

Further successful developments could emerge from serum proteome profiling[40] and from total serum protein glycomics, i.e. the pattern of N-glycans[41]. It was reported that a unique serum proteomic fingerprint is powerful enough (accuracy > 90%) to differentiate between various stages of fibrosis and to allow prediction of fibrosis and cirrhosis in patients with a chronic hepatitis B infection[40]. Specificities, sensitivities and accuracy of prediction of cirrhosis are around 89%. Similarly, N-glycan profiling can distinguish between compensated cirrhosis from non-cirrhotic chronic liver diseases with a sensitivity and specificity of 79% and 86%, respectively[41].

Supplementation of all these laboratory tests by modern high resolution or molecular imaging analyses would be extremely helpful in the consolidation of objective and valid non-invasive biomarkers of diagnosis and follow-up of fibrogenic (liver) diseases. In conclusion, currently available type I and II serum biomarkers should be used with caution, because neither single nor panel markers fulfil the requirements of an ideal non-invasive biomarker of fibrosis[42], i.e. analytical simplicity allowing performance in any laboratory, standardization of the test system and calibrators allowing comparison between laboratories over a long period, cost effectiveness, specificity for the liver and the disease, clear association with the stage of fibrosis or grade of fibrogenesis and independency of the aetiology of fibrosis. Even the best and most extensively evaluated type I (i.e. hyaluronan) and type II (i.e. Fibrotest, Actitest) serum biomarkers do not meet the criteria of an ideal marker. Further detailed insight into the mechanism of liver fibrosis and improvements in analytical techniques will result in new approaches for the non-invasive assessment of fibrosis with biochemical or physical means.

In addition, genetic markers linked with the progression rate of fibrosis will become important diagnostic and prognostic tools for patients with liver fibrosis.

CONCLUSION

Non-invasive evaluation of the fibrogenic response of the chronically injured liver has made considerable progress over the past few years, in particular over the last three years multiple algorithms based on a combination of more or less routine parameters have been suggested frequently. A rigorous, independent and widespread evaluation of the utility of these panels in the diagnosis and follow-up of chronic liver diseases is still needed for a final decision and the recommendation for use in routine clinical practice. Novel single biochemical markers have been suggested, but there putative diagnostic value in clinical practice is far from defined. The fundamental problem in the evaluation of existing and novel non-invasive parameters lies in the limited validity of the present diagnostic "gold standard", i.e. histology of liver biopsy specimens. Perhaps new developments in highly sensitive and tissue-specific scanning techniques of the liver will solve this problem. These procedures will then be suitable for the correct validation of effective antifibrotic treatments.

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EDITORIAL

Inflammatory bowel disease-associated spondyloarthropathies

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Abstract

This issue presents a symposium held in Messina talking about inflammatory bowel disease (IBD) and associated spondyloarthropathies. The topic covers epidemiology and clinical manifestations of IBD-related arthropathies, common genetic and immunologic features, combined therapies for gut and joint inflammation, and future biologic therapies *etc*. I believe this series of articles will deeply facilitate understanding of and the approach to IBD and associated arthropathies.

Key words: Inflammatory bowel disease; Spondyloarthropathies; Anti-tumor necrosis factor α

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In January 2008, a symposium was held in Messina on inflammatory bowel disease (IBD) and associated arthropathies with the aim to improve cooperation between gastroenterologists dealing with IBD and rheumatologists. In an era of highly specialized medicine, it was felt that a better knowledge concerning the clinical features and pathogenic mechanisms of spondylarthritides would lead to an earlier recognition of such extra-intestinal complications among gastroenterologists and, finally, to a more appropriate therapeutic approach involving both specialists.

In the past decades, the relation between IBD and spondyloarthritides has been investigated mainly in epidemiologic studies and in studies concerning HLA-B27 associations. Most studies from the sixties through the eighties focused mainly on axial forms, like ankylosing spondylitis (AS) and sacroiliitis (SI) whereas peripheral manifestations were addressed very much later.

This apparent lack of interest is reflected also by the fact that guidelines on IBD up to 2005 did not mention IBD-associated arthritis. Only in 2006 with the publication of the European Crohn's and Colitis Organization (ECCO) evidence-based consensus on "special situations in Crohn's disease", articular manifestations are briefly discussed[1].

With the introduction of two new topics, genetics and especially biologic therapies, new efforts were made in combination of both kinds of pathology.

Until the end of the former century, only few therapeutic agents like salazopyrin, steroids, and methotrexate were available for the treatment of peripheral arthritis associated with IBD, whereas no disease modifying treatment did exist for axial forms.

With the introduction of the new biologic anti-tumor necrosis factor α (TNF α) therapies, a new chapter for combined treatment options has been opened. Whereas in arthropathies, all available anti-TNF strategies (receptors and antibodies) yielded positive results, in IBD only anti-TNF antibodies, chimeric (Infliximab) or human (Adalimumab), or their fragments (Certolizumab) were shown to effectively down-regulate mucosal inflammation. A possible explanation for the lack of a class effect of anti-TNF strategies may be found in their different mode of action (apoptosis of proinflammatory cells, binding to membrane-bound TNF, *etc*), may be dose-related, or due to immune-related effects from interference with TNF.

In a recent review of studies concerning patients with AS treated with different anti-TNF agents, the odds ratio of flares of an already diagnosed intestinal disease was calculated in comparison with infliximab (odds ratio 1) to be 4.2 for adalimumab and 18 for etanercept[2]. In this paper, a new onset of IBD was reported only for etanercept but not for infliximab or adalimumab.

This TOPIC HIGHLIGHT addresses these different aspects of IBD-associated spondyloarthropathies like epidemiology and clinical manifestations of IBD-related arthropathies, common genetic and immunologic features, diagnostic gastroenterological interventions in pa-

tients with arthritis and endoscopic findings, combined therapies for gut and joint inflammation, and future biologic therapies[3-8]. The papers are written by clinical experts in the field of IBD and experts in the field of gastrointestinal immunology together with their counterparts in the field of rheumatology. The symposium was organized under the auspices of the Italian Society of Gastroenterology (SIGE), the Italian Group for the Study of Inflammatory Bowel Diseases (IG-IBD), and the local academic authorities.

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TOPIC HIGHLIGHT

Walter Fries, MD, *Series Editor*

Gastrointestinal lesions associated with spondyloarthropathies

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Abstract

Subclinical gut inflammation has been described in up to two-thirds of patients with spondyloarthropathies (SpA). Arthritis represents an extra-intestinal manifestation of several gastrointestinal diseases, including inflammatory bowel disease (IBD), Whipple's disease, Behcet's disease, celiac disease, intestinal bypass surgery, parasitic infections of the gut and pseudomembranous colitis. Moreover about two-thirds of nonsteroidal anti-inflammatory drug users demonstrate intestinal inflammation. Arthritis may manifest as a peripheral or axial arthritis. The spondyloarthropathy family consists of the following entities: ankylosing spondylitis, undifferentiated spondyloarthritis, reactive arthritis, psoriatic arthritis, spondyloarthritis associated with IBD, juvenile onset spondyloarthritis. This topic reviews the major gastrointestinal manifestations that can occur in patients with SpA and in nonsteroidal anti-inflammatory drugs users.

Key words: Gastrointestinal diseases; Inflammation; Inflammatory bowel diseases; Crohn's disease; Arthritis; Spondylosis; Ankylosing spondylitis; Nonsteroidal anti-inflammatory drugs

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INTRODUCTION

Arthritis is a recognized extra-intestinal manifestation of several gastrointestinal diseases including inflammatory bowel disease (IBD). Arthritis occurs in 9%-53% of patients with IBD[1-3], and is more prevalent in patients with large bowel disease than in patients with small bowel involvement (42% *vs* 23%)[1]. It may manifest as a peripheral or axial arthritis. Peripheral arthritis includes two different patterns: a pauciarticular arthritis (type 1 arthropathy) striking large joints, which usually accompanies flares of IBD; and a polyarticular arthropathy (type 2 arthropathy) which involves the small joints and is less often associated with flares of IBD[4].

Subclinical gut inflammation has also been described in up to two-thirds of patients with spondyloarthropathies (SpA)[5-9]; histologic gut inflammation was found in SpA in 30%-60% of cases[9]. The observation that the extra-intestinal symptoms generally improve when the gastrointestinal disease is treated, suggests that the association between these two clinical entities is related. The mechanism by which this occurs is not fully understood[1,7].

This topic will review the major gastrointestinal manifestations which can occur in patients having SpA and other diseases with bowel and joint involvement, and in patients having nonsteroidal anti-inflammatory drugs (NSAIDs) related intestinal injuries.

It is useful to begin with a short review of the gastrointestinal function.

GASTROINTESTINAL FUNCTION

The human gastrointestinal tract is not a complete

barrier, being permeable to some macromolecules[10]. Permeability increases in pathologic conditions including IBD[4], celiac disease[11,12], and with the administration of NSAIDs[13,14]. When permeability is increased the gastrointestinal tract is exposed to bacterial and dietary antigens.

The epithelial lining of the gastrointestinal tract includes specialized cells (M cells) which permit transepithelial transport of foreign material from the lumen to mucosal lymphoid tissues; it is evident that some microorganisms use the M cell transport system as a means to infect the mucosa[10,15]. The human intestine harbours a complex microflora composed of aerobic and anaerobic bacteria. In normal individuals antigenic exposure results in tolerance rather than immunity, but this local tolerance is broken in inflamed intestinal tissue. Patients with active IBD lose tolerance to their own bacterial flora, whether this loss of tolerance is a cause or a consequence of the IBD is not known[16-18].

IBD represents a good model for the pathological events that may predispose a host to extraintestinal manifestations. Active IBD is characterized by the following features: (1) A breach of gastrointestinal wall integrity; (2) Increased permeability to macromolecules; (3) Increased exposure to microbial and dietary antigens; (4) Loss of tolerance to own bacterial flora; (5) Host susceptibility to the increased antigenic load. There is also data to suggest that patients with SpA often have subclinical inflammation that may progress to IBD[1].

SpA

The term SpA is used to refer to a family of diseases characterized by inflammation of axial joints, asymmetric oligoarthritis and enthesitis[19]. The SpA family consists of the following entities: ankylosing spondylitis (AS); undifferentiated spondyloarthritis; reactive arthritis (Reiter syndrome); psoriatic arthritis; spondyloarthritis associated with IBD; juvenile onset spondyloarthritis.

The prevalence of SpA in the Caucasian population is 0.5%-2%, with a significant variation worldwide[20].

The need for a standardized approach to classification led to the development of the European Spondyloarthropathy Study Group (ESSG) classification criteria for SpA. According to the ESSG criteria, for a patient to be classified as having SpA he has to show chronic inflammatory back pain before the age of 40 years, persistence for at least 3 mo or asymmetrical synovitis, predominantly of the lower limbs (Table 1). A patient is classified as having spondyloarthritis if he has one or both entry criteria plus one of the following additional criteria: positive family history, psoriasis, IBD, urethritis, cervicitis or acute diarrhea within 1 mo before arthritis, buttock pain alternating between buttocks, enthesopathy or plain film radiographic evidence of sacroiliitis.

The ESSG classification criteria for SpA have been validated in population studies and have a good sensitivity of 75% and a specificity of 87%[21].

An alternative to the ESSC classification criteria is that proposed by Amor and colleagues[22] and considers clinical and historical symptoms, radiologic findings, genetic background and response to treatment (Table 2). These classification criteria are more complicated due to the incorporation of common extra-articular manifestations of disease including gastrointestinal manifestation, but they give improved sensitivity (85%) and specificity (95%). Of these, each criterion is assigned a weight (one or two points) and the resulting points are summed. A patient is considered as having a spondyloarthritis if the sum of the criteria scores is at least six.

A high percentage of patients with SpA (90% of patients with AS and 70% of patients with undifferentiated spondyloarthritis) are HLA-B27 positive. However, this marker is not diagnostic of SpA because a significant percentage of the general population is also positive. In the presence of inflammatory back pain, a positive test for HLA-B27 increases the likelihood of SpA[23].

SpA are associated with several extra-articular manifestations, including acute anterior uveitis[24], genital and skin lesions[25] and inflammatory gut lesions[8].

Table 1 European Spondyloarthropathy Study Group (ESSG) classification criteria for spondyloarthritis

Inflammatory spinal pain, or synovitis (asymmetrical, predominantly in lower limbs), and any one of the following:
Positive family history
Psoriasis
Inflammatory bowel disease
Alternate buttock pain
Enthesopathy

Table 2 Amor classification criteria for spondyloarthropathy

Clinical symptoms or past history of
Lumbar or dorsal pain at night, or lumbar or dorsal morning stiffness = 1
Asymmetrical oligoarthritis = 2
Buttock pain (buttock pain = 1, alternating buttock pain = 2)
Sausage-like finger or toe = 2
Heel pain = 2
Iritis = 2
Non-gonococcal urethritis or cervicitis accompanying, or within 1 mo before, the onset of arthritis = 1
Acute diarrhoea accompanying, or within 1 mo before, the onset of arthritis = 1
Presence of history of psoriasis and/or balanitis and/or of inflammatory bowel disease (ulcerative colitis, Crohn's disease) = 2
Radiological findings
Sacroiliitis (grade > 2 if bilateral, grade > 3 if unilateral) = 3
Genetic background
Presence of HLA-B27 and/or family history of ankylosing spondylitis, reactive arthritis, uveitis, psoriasis or chronic inflammatory bowel disease = 2
Response to therapy
Definite improvement of musculoskeletal complaints with NSAIDs in less than 48 h or relapse of the pain in less than 48 h if NSAIDs discontinued = 2
A patient is considered as having a spondyloarthropathy if the sum of the scores is 6 or more

Table 3 Histologic types of gut inflammation in patients with SpA

Acute	Chronic
Architecture preserved	Architecture disturbed
PMN infiltration	Irregular, blunted and fused villi
Granulocytes, lymphocytes, plasma cells in lamina propria	Distortion crypts
	Basal lymphoid follicles
	Sarcoid-like granulomas

Two histologic types of gut inflammation in patients with SpA can be distinguished: acute and chronic inflammation (Table 3). This classification refers to the morphologic characteristics and not to the onset or duration of the disease.

The acute type mimics the acute bacterial enterocolitis: the mucosal architecture is well preserved, there is a polymorphonuclear infiltration of the ileal villi and crypts, and an increased number of inflammatory cells (granulocytes, lymphocytes and plasma cells) in the lamina propria.

Acute lesions are mainly seen in patients with reactive arthritis.

The chronic type is often indistinguishable from Crohn's disease (CD): the mucosal architecture is clearly disturbed, the villi are irregular, blunted and fused; the crypts are distorted and the lamina propria is edematous and infiltrated by mononuclear cells. In some cases aphthoid ulcers and sarcoid-like granulomas are present. Chronic lesions are more present in undifferentiated SpA and AS[9].

Similarities are present between the immune alterations in SpA and CD, suggesting that these are probably distinct phenotypes of a common immune-mediated disease, possibly being expressed in a genetically different host; in fact, the mutations of the CARD15/NOD2 gene that have been associated with CD have not been found in AS[26]. These similarities are: an increased expression of α-E-β-7 in T-cells from patients with SpA and in the intestinal lymphocytes of patients with CD; an increased expression of epithelial A-cadherin; an increased expression of CD 163 positive macrophages in CD and SpA; relative contribution of T-helper 1 cells; presence of IgA antibodies to Saccharomyces cerevisiae.

According to current knowledge, there is a clinical relationship between gut and joint inflammation in SpA, and the gut could have an important pathogenic role[9]: the prevalence of gut inflammation in AS is higher in patients with associated peripheral arthritis than in patients without arthritis[6]; chronic lesions in the gut are associated with more advanced radiologic signs of sacroiliitis and spondylitis and with more destructive peripheral arthritis[27]; remission of joint inflammation is usually associated with a disappearance of gut inflammation, whereas the persistence of locomotor inflammation is mostly associated with the persistence of gut inflammation[28-31].

Clinical, genetic, histopathologic and immunologic data suggest that SpA and CD probably should be considered as distinct phenotypes of a common immune-mediated inflammatory disease pathway rather than as separate disease entities[9].

Patients with peripheral arthritis and AS are often found to have endoscopic and histologic signs of small bowel inflammation, and a fraction of these patients go on to develop clinically overt CD. Moreover, some patients with SpA have a form of sub-clinical CD in which locomotor inflammation is the only clinical expression. In a prospective long-term study at first investigation about 6% of patients with SpA did not present any sign of CD, but demonstrated gut inflammation on biopsy. They developed CD 2 to 9 years later[32].

Ileal and colonic mucosal ulcerations in patients with SpA can be detected by endoscopy. Endoscopic lesions were found in 44% of patients with SpA versus 6% of patients with other inflammatory arthritis and the most common endoscopic diagnosis was early CD (26%)[5].

It has been highlighted that a capsule endoscopy can provide important information on upper gastrointestinal pathology in patients with SpA in which there is small bowel involvement. Eliakim *et al*[33] have compared the diagnostic yield of capsule endoscopy with that of ileo-colonoscopy in the finding of small bowel lesions in patients with SpA; significant small bowel findings (erythema, aphthous, erosions) were detected by capsule endoscopy in 30% and by ileo-colonoscopy in only 9% patients with SpA.

The association between SpA and clinical or subclinical intestinal association has rarely been described in children. Conti *et al*[34] investigated a group of 129 children for suspected IBD, 31 of whom had signs of axial and/or peripheral arthropathy, and after ileo-colonoscopy with biopsy, 7 children had classic IBD, 12 had indeterminate colitis, and 12 had lymphoid nodular hyperplasia of the distal ileum as the main feature. All were HLA-B27 negative. These patients may be a population at risk of developing a full IBD phenotype. SpA may be the initial manifestation of systemic disorders such as IBD.

Ankylosing spondylitis

AS is the most common disease among the SpA; it is a chronic inflammatory disease of the axial skeleton characterized by back pain and progressive rigidity of the spine. AS usually affects young adults and an association with the human leukocyte antigen HLA-B27 was observed. The association with HLA-B27 is weaker in IBD-associated AS than in idiopathic AS. Radiographic changes of the hips are present in roughly 10% of patients[35]. Other organs, such as eyes, lungs, gut and heart can be affected. Up to two-thirds of patients with AS have subclinical gut inflammations shown either by endoscopy or histology, between 5% and 10% of cases of AS are associated with IBD. Despite these observations, systematic screening of AS patients by ileo-colonoscopy is not indicated in the absence of gut symptomatology as only a small proportion of AS patients with subclinical gut inflammation will develop IBD in the future[2].

Reactive arthritis

Reactive arthritis may occur after an enteric infection due to *Salmonella*, *Shigella*, *Yersinia* or *Campylobacter species* with an incidence ranging from 2% to 33%. In particular an increased risk of arthritis is associated with a *Yersinia* infection and the presence of the HLA-B27 genotype. Joint symptoms develop within 2-3 wk of developing diarrhea and involve knee, ankle, wrist, and sacroiliac joints. To confirm the clinical suspicion of reactive arthritis it is useful to demonstrate a pathogenic organism by stool culture or a rise of antibody titres. Antibiotic treatment may be effective during the diarrheal phase but not when arthritis is present[8,27,36].

OTHER DISEASES WITH BOWEL AND JOINT INVOLVEMENT

In addition to ulcerative colitis and CD, other illnesses have intestinal involvement and arthritis as prominent clinical features. These include Whipple's disease, Behcet's disease, celiac disease, intestinal bypass arthritis, parasitic rheumatism, and pseudomembranous colitis. These disorders are also considered in the differential diagnosis of patients with suspected IBD and arthritis.

Whipple's disease

Whipple's disease is due to an infection with *Tropheryma Whippelii* and may cause diarrhea, malabsorption and weight loss. Systemic infection is often associated with joint manifestations, involving the knee, the ankle and the wrist and sometimes it is associated with spondylitis and sacroiliac joint involvement. In some patients the articular symptoms develop prior to symptomatic enteric involvement. A small bowel biopsy is usually diagnostic. Whipple's disease requires long term antibiotic therapy[37].

Behcet's disease

Behcet's disease is characterized by oral and genital ulceration, iritis and occasionally by central nervous system involvement; oligoarticular, asymmetric arthralgia and arthritis may develop in 50% of patients involving the knee, the ankle, the wrist and the elbow. Mucosal ulceration of the small bowel is a frequent manifestation of Behcet's disease and may cause nausea, diarrhea, abdominal pain and distension. It is often difficult to distinguish from IBD[38,39].

Celiac disease

Celiac disease may be associated with arthritis in some patients; articular involvement was peripheral in 10%, axial in 8% and combined in 9%. The arthritis is typically non erosive and can be either oligo-or-polyarticular. Joint symptoms may precede gastrointestinal manifestations and respond to a gluten-free diet[11,12].

Intestinal bypass surgery

Intestinal bypass surgery is a surgical technique used for the treatment of obesity in the past. Arthritis affecting knee, wrist, ankle, shoulder and finger joints was recognized as a postoperative complication of this technique. Polyarthralgia and sometimes arthritis has been reported to occur weeks or years following surgery in 8% to 36% of patients[2,36].

Parasitic infections

Parasitic infections of the gut due to *Strongyloides stercoralis*, *Taenia saginata*, *Endolimax nana* and *Dracunculus medinensis* have been associated with a form of reactive arthritis[36].

Pseudomembranous colitis

Arthritis associated with pseudomembranous colitis has been described following antibiotic therapy, often affecting the large joints of the lower extremity. In a report of four patients, arthritis developed 9-35 d after the onset of diarrhea[36].

NSAIDS RELATED INTESTINAL INIURY

The distal small bowel and colon are susceptible to the dangerous effects of NSAIDs; although the proportion of patients who develop clinically important NSAID-induced enteropathy or colopathy is small, the intestinal injuries induced by NSAIDs, including erosions, ulcers, strictures and perforations, are common[13]. About two-thirds of NSAID users demonstrate intestinal inflammation[13,14,40]. In a case control study, patients with small or large bowel perforation or bleeding were more than twice as likely to be NSAID users[41]. In an autopsy study nonspecific small intestinal ulceration was much more common in those who had taken NSAIDs (8.4% *vs* 0.6%)[42]. A randomized, controlled trial revealed more mucosal breaks in a group of patients who used NSAIDs plus omeprazole compared with a group of patients who used COX-2 inhibitors. This study suggests relative protection of the COX-2 inhibitors compared with non-selective NSAIDs plus omeprazole against small bowel injury[43]. By contrast a non-randomized cohort study found similar rates and types of small bowel injury with long-term use of COX-2 selective agents versus NSAIDs[44].

Most NSAID-induced injuries are subclinical and go unrecognized. When present, symptoms and signs are nonspecific and may include: anaemia, bleeding from ulcers, hypoalbuminemia, intermittent or complete bowel obstruction from broad-based or diaphragm-like strictures, watery or bloody diarrhea and acute abdomen. The typical patient is one taking a NSAID for a rheumatic condition and the duration of NSAID use to time of diagnosis is widely variable (days to years)[41].

A pathognomonic lesion of NSAID injury is the diaphragm-like stricture, which is likely to be due to a scarring reaction secondary to ulcerative injury. These lesions are thin, concentric, diaphragm-like septa which are usually multiple in the mid-intestine but may be also present in the ileum and colon. Histologically, they are characterized by sub-mucosal fibrosis with normal overlying epithelium, apart from the tip of diaphragm,

which may be ulcerated; the mucosa between diaphragms is normal[14,45].

Capsule endoscopy, double-balloon enteroscopy and colonoscopy may help in the diagnosis of NSAID-induced injury, although there is nothing endoscopically specific about NSAID-induced gut lesions[46,47]. Histology is also nonspecific; the differential diagnosis should include: Campylobacter, Yersinia, Cytomegalovirus, TB infections, IBD, ischemia, radiation enteritis, vasculitides and other drugs. The NSAIDs-induced lesions generally improve upon withdrawal of the drug.

CONCLUSION

An important role of the gut in the pathogenesis of SpA and for an overlap between SpA and CD is supposed. It is not clear if SpA and CD should be considered as distinct phenotypes of common immune-mediated inflammatory disease pathways or separate disease entities. Systematic screening of SpA patients by ileo-colonoscopy is not indicated in the absence of gut symptomatology but these patients may be a population at risk of developing a full IBD phenotype. It is important to know that there are other diseases with bowel and joint involvement like Whipple's disease, celiac disease and so on and that two-thirds of NSAID users demonstrate intestinal nonspecific inflammation.

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TOPIC HIGHLIGHT

Walter Fries, MD, *Series Editor*

Clinical features and epidemiology of spondyloarthritides associated with inflammatory bowel disease

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Abstract

Inflammation of axial and/or peripheral joints is one of the most frequent extra-intestinal manifestations complicating the clinical course and therapeutic approach in inflammatory bowel diseases (IBD). The frequency of these complications seems to be similar for both diseases, Crohn's disease and ulcerative colitis. Arthritis associated with IBD belongs to the category of spondyloarthropathies. Axial involvement ranges from isolated inflammatory back pain to ankylosing spondylitis, whereas peripheral arthritis is noted in pauciarticular and in polyarticular disease. Asymptomatic radiological involvement of the sacroiliac joints is reported to occur in up to 50% of patients. Other musculoskeletal manifestations such as buttock pain, dactylitis, calcaneal enthesitis, and thoracic pain are frequently underdiagnosed and, consequently, are not treated appropriately. Several diagnostic approaches and criteria have been proposed over the past 40 years in an attempt to correctly classify and diagnose such manifestations. The correct recognition of spondylarthropathies needs an integrated multidisciplinary approach in order to identify common therapeutic strategies, especially in the era of the new biologic therapies.

Key words: Crohn disease; Ankylosing spondylitis; Ulcerative colitis; Spondylarthropathies; Arthritis; Enthesopathy

INTRODUCTION

Arthritis, belonging to the category of spondylarthropathy, is the most frequent extra-intestinal complication of inflammatory bowel diseases (IBD). The clinical spectrum of spondylarthropathies includes axial symptoms, peripheral arthritis, dactylitis and enthesopathy. Musculoskeletal manifestations occur in 20%-50% of patients with IBD[1-3].

Spondyloarthropathies (SpA), or spondyloarthritides as recently proposed[4], represent a group of distinct diseases with similar clinical features and a common genetic predisposition. The 5 major subtypes are ankylosing spondylitis (AS), psoriatic arthritis, reactive arthritis, IBD-associated SpA (IBD-SpA), and undifferentiated SpA. The main recognized genetic association is with HLA-B27, but it is clear that there are other genes involved.

According to the European Spondyloarthropathy Study Group (ESSG) criteria[5], IBD is a criterion of spondylarthropathy; psoriasis or enteric infections are 2 other manifestations included in the ESSG criteria for identifying patients with psoriatic arthritis and reactive arthritis. Thus, patients with IBD presenting with inflammatory back pain and/or synovitis (predominantly of the lower limbs) are diagnosed as having spondyloarthropathy (Table 1). The ESSG criteria, designed to be applicable without radiological examination and laboratory testing, have good sensitivity (75%) and specificity (87%), at least in established disease. An alternative classification scheme was put

forward by Amor *et al*[6] (Table 2), which is more complicated but gives improved sensitivity (85%) and specificity (90%) due to the incorporation of common extra-articular manifestations of disease, including enthesopathy, dactylitis, eye disease and HLA-B27 positivity. The basic concepts underlying each classification set are nevertheless similar.

CLINICAL FEATURES

The clinical picture of IBD-SpA is characterized by axial and/or peripheral joint involvement in the absence of rheumatoid factors and of typical extra-articular findings of rheumatoid arthritis (e.g. subcutaneous nodules)[7].

Axial joint disease

The spectrum of axial involvement ranges from inflammatory lower back pain with or without radiological evidence of sacroiliitis (SI), asymptomatic SI, and overt AS characterized by the "classical" clinical (spine stiffness, pain) and radiologic features (squaring, syndesmophytes, bamboo spine).

Inflammatory back pain (IBP)

IBP is usually difficult to localize, insidious in onset, frequently monolateral and intermittent at onset, more intense at rest, associated with stiffness but relieved by movement, exacerbated by cough or sneezing, and accompanied by fatigue. The diagnosis is clinical and defined according to Calin's criteria[8] (Table 3), based on morning stiffness, age and modality of onset, and duration. The diagnosis of IBP is based on the positivity of at least 4 out of 5 parameters. A modification of these criteria with a higher sensitivity has been recently proposed and, apart from the duration of pain and morning stiffness, also considers improvement with exercise, awakening because of pain, and the presence of alternating buttock pain[9].

According to this modification a 70% sensitivity and an 81% specificity are achieved if at least 2 parameters are fulfilled with a positive likelihood ratio 3.7; if at least 3 are fulfilled, the positive likelihood ratio raises to 12.4.

In the presence of IBP, the radiologic evaluation of the sacroiliac joints allows to make diagnosis of SI. SI is graded according to the radiologic criteria established since 1966[10] (Table 4). In the absence of findings with conventional X-ray examination, magnetic resonance imaging (MRI) evaluation may allow diagnosis and, thus, effective early treatment for axial spondyloarthritis[11]. The evidence of an increased signal in the bone and bone marrow (bone edema) with T1 post-gadolinium and STIR (short tau inversion recovery) techniques is a sign of active inflammation in the SI joints and/or spine (Figure 1).

The importance of an early diagnosis has been underlined in a study carried out on 25 HLA-B27 positive patients with IBP and a grade 2 (or lower) unilateral SI on conventional radiography. In these patients, when studied by MRI, 36/50 joints were diagnosed as having grade 2 or higher SI, and bone edema was found in 20/50. The same patients were studied 3 years later by conventional radiography and demonstrated grade 2 or more SI in 21/44 sacroiliac joints, with the conclusion that MRI is more sensitive than conventional radiography for the detection of SI in the early stages[12].

The diagnostic criteria for classic AS have been subjected to several changes over the past decades from the Rome Criteria[13] to the New York Criteria[7] and then to the Modified New York Criteria[14] in 1984 (for more detail concerning evolution of diagnostic criteria)[15]. This

Table 1 Diagnostic criteria according to the ESSG[2]

Inflammatory spinal pain or/and synovitis asymmetric or predominantly of the lower limbs
One or more of the following
Positive family history
Inflammatory bowel disease
Urethritis, cervicitis, or acute diarrhea within 1 mo before arthritis
Buttock pain alternating between right left gluteal areas
Enthesopathy
Sacroiliitis

Table 2 Amor diagnostic criteria for spondylarthropathy[3]

Clinical symptoms or past history of
Lumbar or dorsal pain at night, or lumbar or dorsal morningstiffness = 1
Asymmetric oligoarthritis = 2
Buttock pain (buttock pain = 1, alternating buttock pain = 2)
Sausage-like finger or toe = 2
Heel pain = 2
Iritis = 2
Non-gonococcal urethritis or cervicitis accompanying, or within 1 mo before, the onset of arthritis = 1
Acute diarrhea accompanying, or within 1 mo before, the onset of arthritis = 1
Presence or history of psoriasis and/or balanitis and/or of inflammatory bowel disease (ulcerative colitis, Crohn's disease) = 2
Radiological findings
Sacroiliitis (grade > 2 if bilateral, grade > 3 if unilateral) = 3
Genetic background
Presence of HLA-B27 and/or family history of ankylosing spondylitis reactive arthritis, uveitis, psoriasis or chronic inflammatory bowel disease = 2
Response to therapy
Definite improvement of musculoskeletal complaints with NSAIDs in less than 48 h or relapse of the pain if NSAIDs are discontinued = 2
A patient is considered as having spondylarthropathy if the sum of the scores is 6 or more

Table 3 Calin's criteria for the clinical diagnosis of inflammatory back pain[5]

Onset before age 45 yr
Insidious onset
Improvement with exercise
Morning stiffness
Persistence (at least 3 mo)
Coexistence of 4 out of 5 criteria allows the definition of inflammatory back pain

Table 4 Radiologic criteria for staging of inflammatory changes related to sacroiliitis[7]

Sacro-iliac joints	
Grade 0	Normal
Grade 1	Suspicious changes
Grade 2	Minimal abnormality-small localized areas with erosions or sclerosis without alterations in joint width
Grade 3	Unequivocal abnormality-moderate or advanced sacro-iliitis with one or more of the following: erosions, sclerosis, widening
Grade 4	Severe abnormality-total ankylosis

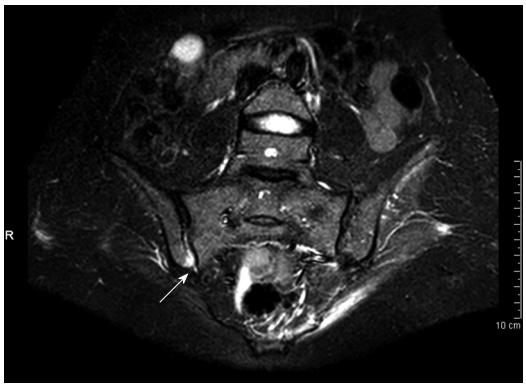

Figure 1 MRI of the sacroiliac joints of a 39-year-old patient with inflammatory back pain and ulcerative colitis (pelvis radiograph was negative for sacroiliitis): STIR MRI of the sacroiliac joints shows bone marrow edema (arrow) of the right sacroiliac joint.

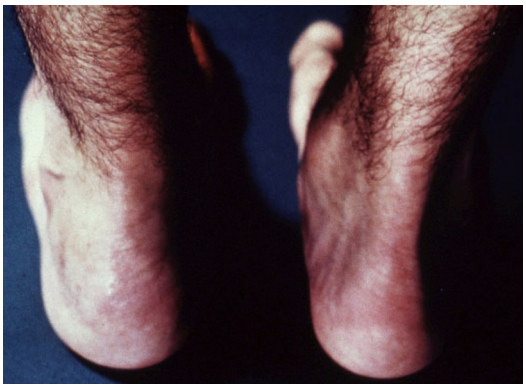

Figure 2 Achilles tendonitis due to enthesitis of the tendon insertions.

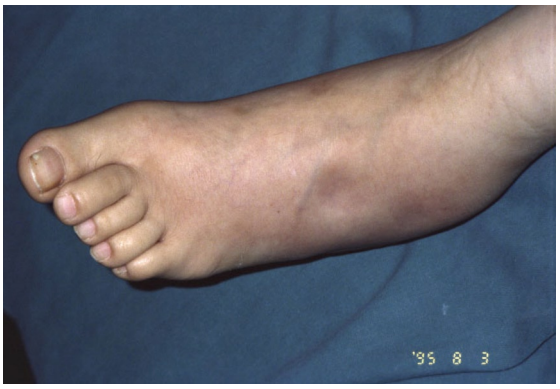

Figure 3 Dactylitis with sausage-like appearance of the Ⅱ toe.

latter classification is based on: (1) lower back pain of at least 3 mo duration that improved with exercise and was not relieved by rest; (2) limited lumbar spinal motion in sagittal (sideways) and frontal (forward and backward) planes; (3) decrease of chest expansion, assessed at the Ⅳ intercostal space, relative to normal values for sex and age; and (4) bilateral SI grade 2-4 or unilateral SI grade 3 or 4. A definite diagnosis of AS is made if criterion 4 (radiology) and any one of the other criteria are fulfilled.

Apart from radiologic alterations, one of the key features of AS is the presence of IBP and/or alternate buttock pain. Other clinical signs to search for in order to assess reduced spine mobility include the Schober test, finger to floor distance, maneuvers for cervical spine assessment (occiput to wall, tragus to wall, cervical rotation), and investigation in order to establish a reduced chest expansion (for review see the INSPIRE study)[16].

Peripheral arthritis

The recognition of this entity is based on clinical diagnosis, e.g. joint swelling and tenderness but may be confirmed by ultrasound examination[17] or MRI[18], whereas conventional radiographs usually are not helpful.

Two subtypes of peripheral arthritis are recognized[1]. Type 1 involves less than 5 joints and is clinically characterized by acute self-limiting attacks of less than 10 wk duration often paralleling intestinal inflammatory activity. Moreover, it is strongly associated with other extra-intestinal manifestations of IBD such as erythema nodosum. Type 2 peripheral arthritis is polyarticular, involving 5 or more joints with symptoms that persist for months and years running independently from IBD flares. This type is associated with uveitis but not with other extra-intestinal manifestations. Both types are seronegative, usually non-erosive and non-deforming, but may become chronic and erosive in 10% of patients[19]. In addition, no significant association has been shown between peripheral arthropathies and HLA-B27 in IBD[1,19-21].

A type 3 peripheral arthritis has been proposed, which includes patients with both axial involvement and peripheral arthritis[22].

Other manifestations

Enthesitis is inflammation at the site of the tendon, ligament and joint capsule insertion to bone. The most frequent clinical expressions are Achilles tendonitis (Figure 2), plantar fasciitis and/or pain and swelling of the tibial tubercle[23]. Diagnosis is clinical but may be confirmed by ultrasound[24] or MRI[25].

Dactylitis (Figure 3) is characterized by the inflammatory swelling of one or more fingers (sausage fingers) or toes caused by tenosynovitis of the flexor tendons. Metacarpophalangeal or proximal interphalangeal arthritis may be associated.

Thoracic pain results from enthesitis of costovertebral,

Table 5 Early epidemiologic studies on IBD-associated arthropathies

Author	Yr	Population	Patients	AS (%)	SI (%)	Peripheral arthritis (%)	HLA-B27 (%)
Acheson[27]	1960	USA	CD 742	2.3	NA	NA	NA
			UC 1175	2.0	NA	NA	NA
Ansell[28]	1964	Canada	CD 91	6.5	19.7	15.3	NA
Haslock[29]	1973	Great Britain	CD 116	16	NA	20.4	NA
Wright[30]	1965	Great Britain	UC 234	6.4	17.9	NA	NA
Wright[31]	1965	Great Britain	UC 269	5.5	NA	11.5	NA
Dekker-Saeys[20]	1978	Netherlands	CD 51	3.9	15.6	11.7	3.9
			UC 58	3.4	12.0	14.0	18.9
Rankin[32]	1979	USA	CD 569	NA	NA	19.0	NA
Műnch[33]	1986	Germany	CD 167	9.0	20.0	14.0	5.3

NA: Not available; IBD: Inflammatory bowel diseases; CD: Crohn's disease; UC: Ulcerative colitis; SI: Sacroiliitis.

Table 6 Epidemiologic studies on mixed IBD populations unless otherwise indicated

Author	Yr	Country	Patients	AS[1] (%)	SI (%)	Peripheral arthritis (%)	IBD-SpA[2] (%)	IBP[3] (%)	Enthesopathy (%)	Overall (%)
Scarpa[34]	1992	Italy	79 (UC)	25.3	43	18.9	-	-	-	62
Protzer[35]	1996	Germany	521	45.1	-	28.1	11.5	-	-	-
Veloso[36]	1996	Portugal	792	3.0	-	16.2	-	-	-	-
Orchard[1]	1998	Great Britain	1459	1.0	-	7.4	-	5.2	-	21.4
Suh[4][37]	1998	Korea	129	1.6	6.2	15.5	-	-	-	17.1
De Vlam[38]	2000	Netherlands	103 (CD)	3.8	21.8[4]		34.9	30	7	39
Queiro[39]	2000	Spain	62 (UC)	3.2	24.2	30.6	-	-	-	-
Salvarani[2]	2001	Italy Netherlands	160	2.6	3.6	10.6	18.1	8.8	10	33.1
Christodoulou[5][40]	2002	Greece	252	-	5.9	2.8	-	-	-	17.0
Palm[6][41]	2002	Norway	406	2.4	2.0	17	22	18.0	26	32.5
Mendoza[42]	2005	Spain	566	1.8	1.9	6.7	-	-	-	-
Turkcapar[3]	2006	Turkey	162	9.9	45.7	14.8	45.7	-	50.0	-
Peeters[43]	2008	Belgium	251 (CD)	6	27	29	-	-	-	-
Rodriguez[44]	2008	Puerto Rico	100	2.6	13	5	42	42	-	-
Lanna[45]	2008	Brazil	130	6.2	9.2	25.4	-	10	5.4	31.5

[1]Modified New York criteria; [2]IBD-SpA according to ESSG criteria; [3]Calin's criteria; [4]Classification according to Gravallese[62]; [5]Only symptomatic SI, X-ray assessment; Undefined arthralgia group; no definitions are given for spondylitis or arthritis; [6]IBP and SI were considered excluding patients with AS; asymptomatic patients included in SI.

costosternal, manubriocostal articulations, exacerbates with cough and deep inspirations, limits respiratory expansion, and episodes are of variable duration.

Buttock pain is part of the IBP, irradiates to the sacrum and may be alternating; it is related to inflammation of sacroiliac joints.

Extra-articular features are represented by uveitis (25%), aortic insufficiency (4%-10%), and cardiac conduction disturbances 3%-9%[26]. These latter cardiologic complications seem to be related to disease duration and are associated with HLA-B27.

EPIDEMIOLOGY

With respect to the evolution of diagnostic criteria, studies on IBD populations from the 1960s, 70s and 80s include patients with axial joint involvement with application of restricted criteria substantially mirroring classic AS or SI together with peripheral arthritis. Table 5 summarizes the principal data from those early studies[20,27-33]. From these studies AS was found to be present in 2% to 16% of patients with higher numbers for Crohn's disease (CD) compared to ulcerative colitis (UC). Asymptomatic and symptomatic SI was found in 12% to 20% of patients and peripheral arthritis in 11% to 20%. Association with HLA-B27 ranged from 3.9% to 18.9%.

Studies on IBD populations after the introduction of the ESSG criteria or Amor criteria are summarized in Table 6[1,34-45]. A discrete number of papers reporting on IBD-associated joint disease were not included. Most of the excluded studies aimed to detect the frequency of every kind of extra-intestinal manifestation of IBD and were not specifically directed to identify IBD-SpA lacking exact definitions of diagnostic criteria. So, Maeda *et al*[46] found that out of 203 Japanese CD patients, 21 had arthritis (10.3%) and 3 had spondylitis (1.5%). Triantafillidis *et al*[47] reported a frequency of 30% of arthritis/arthralgias in a cohort of 155 Greek CD patients. The study by Bernstein *et al*[48] from Canada was based on the ICD code from hospitalized patients with IBD reporting a 4% prevalence of AS with male CD patients being more frequently affected than male UC patients. Souza *et al*[49], in a mixed Brazilian IBD population found a prevalence of 14.4%, with no difference between CD and UC. Al-Shamali *et al*[50] reported

an 8.9% prevalence of arthritis in UC patients from Kuwait with an overall prevalence of rheumatologic complaints of 31%.

Other studies aimed to identify the frequency of symptomatic or asymptomatic SI. Steer *et al*[51] found on CT examination 31/134 of CD patients, symptomatic for back pain, signs of SI (16 of these patients were missed by conventional X-ray). In another study carried out in 50 CD patients symptomatic for back pain, 28% fulfilled the modified NY criteria for AS on X-ray examination[52]. On the other hand, asymptomatic SI may be present in 10% to 50% of patients with IBD[19]. In a comparative study employing conventional X-ray and CT, changes compatible with SI were found in 29% of CD patients being symptomatic only 3%[53].

In the studies included in Table 6, overall prevalence of any manifestation ranged from 17% to 62%. AS ranged from 1% to 25.3%, SI from 1% to 45.7%, peripheral arthritis from 2.8% to 30.6%, IBD-SpA according to the ESSG criteria from 5% to 45.7%, and IBP in 5.2% to 42%. Other manifestations such as inflammatory enthesopathies, when present, were found in 7% to 50% of patients.

With regard to differences between CD and UC, most studies reported similar figures for peripheral and axial involvement in both pathologies. Concerning disease localization, most studies agreed that ulcerative proctitis is rarely complicated by joint inflammation and, concerning CD, that inflammatory joint disease occurs with increased frequency in Crohn's colitis compared to ileal involvement. A discrete percentage of patients will develop one or more spondylarthropathy-related manifestations (such as isolated calcaneal enthesitis and/or dactylitis)[39] without fulfilling any of the classification criteria.

Whereas type I peripheral arthritis is associated with intestinal disease activity[1], SI, especially in its asymptomatic form, is equally present in CD and UC[38,54], and seems more related to duration of IBD. Taken together, SI is one of the most frequent joint inflammations found in IBD patients[39]. The onset of axial symptoms may precede the diagnosis of intestinal disease by decades.

HLA-B27

The importance of HLA-B27 in conferring susceptibility to AS is well known, although the molecular basis is not completely understood. The HLA-B27 gene is located on the short arm of chromosome 6 and comprises 31 proteins with HLA-B*2705, 02, 04, and HLA-B*2707 as the major subtypes associated with disease. Several hypotheses are discussed on how HLA-B27 works on a molecular level in mediating joint inflammation. The arthritogenic peptide hypothesis postulates that HLA-B27 specific receptors on $CD8^+$ T-cells recognize antigenic peptides and subsequently elicit a cytotoxic T-cell mediated autoimmune response. The misfolding hypothesis states that an aberrant folding of HLA-B27 heavy chains occurs in the endoplasmic reticulum leading to a misfolded B-pocket of the peptide-binding groove and hyperaccumulation leading finally to cytokine and chemokine transcription. A third hypothesis suggests sharing of homing receptors on gut epithelium and synovium together with an impaired elimination of intracellular bacteria by HLA-B27 to represent the base for joint inflammation[55].

The prevalence of HLA-B27 varies greatly in the different ethnicities ranging from 0% in African Bantu and Australian Aborigines to 50% in Native Americans[56]. The prevalence in Western European countries varies from 3% to 18%. In Western European populations, HLA-B27 is found in 90% of patients with AS, in 30%-70% of patients with reactive arthritis, in approx 70% of undifferentiated SpA, in 50% of acute anterior uveitis and in 88% of patients with heart block associated with aortic insufficiency.

The association between axial involvement and HLAB27 in IBD patients is much less conclusive: only 25%-75% of patients with CD and AS present positivity for HLA-B27[34,57,58]. Pure asymptomatic SI in CD is not strongly associated with HLA-B27 and a very recent study indicates a prevalence of 7% (comparable to prevalence in the healthy population)[43], and it seems that evolution to AS is more likely to occur in HLAB27-positive patients[59]. This suggests that SI and AS in IBD patients are different entities. A similar distinction has been proposed for peripheral arthritis. Recently, Orchard *et al*[60] have observed an association with HLA-DRB1*0103, B*35 and B*27 in type 1 peripheral arthritis. Similar associations were observed in a control group consisting of patients with postenteric reactive arthritis. Neither HLA-B27 nor DR-4 associations were observed in type 2 arthropathy. These data indicate that type 1 and 2 arthropathies are immuno-genetically distinct entities and that type 1 is more similar to axial spondyloarthropathies

HLA-B27 testing as a tool for achieving diagnosis is useful only in patients with high pre-test probability and thus its use as a screening test is not recommended. In patients with clinically assessed presence of IBP (14% probability of axial SpA), HLA testing may follow and a positive test result would mandate a subsequent referral to a rheumatologist for further evaluation because the probability of axial SpA in such a patient would increase to 59%[61].

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TOPIC HIGHLIGHT

Walter Fries, MD, *Series Editor*

Enteropathic spondyloarthropathy: A common genetic background with inflammatory bowel disease?

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Abstract

The association between spondyloarthropathy and inflammatory bowel disease (IBD) is largely established, although prevalence is variable because of different population selection and diagnostic methodologies. Most studies indicate that as many as 10%-15% of cases of IBD are complicated by ankylosing spondylitis (AS) or other forms of spondylarthritis (SpA). Of note, ileal inflammation resembling IBD has been reported in up to two thirds of cases of SpA, and it has been suggested that the presence of ileitis is associated with the chronicity of articular complications. Although this observation is of interest to unravel the pathophysiology of the disease, systematic screening of patients with SpA by ileocolonoscopy is not indicated in the absence of gut symptoms, as only a small proportion of patients with subclinical gut inflammation will develop overt IBD over time. The existence of familial clustering of both IBD and AS, the coexistence of both conditions in a patient, the evidence of an increased risk ratio among first- and second-degree relatives of affected AS or IBD patients and finally, the increased cross-risk ratios between AS and IBD, strongly suggest a shared genetic background. So far, however, IL23R is the only identified susceptibility gene shared by both IBD and AS. Although functional studies are still needed to better understand its pathogenic role, great effort is being spent therapeutically targeting this pathway that may prove effective for both disorders.

Key words: Inflammatory bowel disease; Ulcerative colitis; Crohn's disease; Ankylosing spondlitis; Sacroiliitis; Spondyloarthropathy

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INTRODUCTION

Crohn's disease (CD) and ulcerative colitis (UC) are chronic relapsing inflammatory bowel diseases (IBDs) of unknown etiology that affect up to 1 in 250 of the adult population, with up to 25% of patients being diagnosed during childhood or adolescence[1]. Key features of UC include diffuse mucosal inflammation that extends proximally from the rectum. In CD, conversely, any site in the gastrointestinal tract may be affected with transmural inflammation, which is typically patchy and segmental[2]. Joint complications are the most common extra-intestinal manifestations of IBD, and were recognized as far back as the 1920s. Both axial and peripheral joint complications are recognized. This review mainly focuses on the so-called enteropathic spondyloarthropathy, and more specifically, on the possible pathogenic link which may lie in a common genetic background with IBD.

ANKYLOSING SPONDYLITIS (AS)

AS is a seronegative inflammatory arthropathy that affects

the vertebral column, and is characterized by sacroiliitis and progressive ankylosis (fusion) of the vertebral facet joints. It is defined classically by the modified New York Criteria[3], which include: low back pain for more than 3 mo; relieved by exercise and not improved by rest; limited spinal movement in two planes; and decreased chest expansion. If any of these clinical features is present in association with bilateral grade 2-4 or unilateral grade 3-4 sacroiliitis, then a definitive diagnosis can be made. However, there is some overlap with the more general term of sponyloarthropathy (SpA), as defined by the European Study Group on Spondyloarthropathy[4]. This involves a combination of inflammatory low back pain and a number of other factors including associated conditions, such as IBD or enteric infection, post-dysenteric reactive arthritis, psoriatic arthritis, and post-urethritis arthritis. The prevalence of AS in the general population is 0.25%-1%[5,6], but it is increased in IBD patients to 1%-6%, depending on the study population and the method of investigation[7,8]. In a recent population-based study in Italy[9], the prevalence of AS was 0.37%, while IBD-associated arthritis accounted for 0.09%. However, the clinical spectrum might be broader than that so far defined. In a population-based inception cohort of IBD patients evaluated in Italy and Netherlands, 18% satisfied the European criteria for SpA, 3.1% satisfied the modified New York criteria for AS, however, 14.4% patients developed one or more SpA-related manifestations, without fulfilling any of the classification criteria[10].

The traditional method of radiological assessment is by plain radiology of the sacroiliac joints. Magnetic resonance imaging has demonstrated a higher sensitivity[59], although in the absence of symptoms, its significance is sometimes unclear. Idiopathic AS is more common in male subjects, with an M:F ratio of 3:1, whereas in IBD, the M:F ratio is closer to 1:1. The main lesion of AS is sacroiliitis and this is associated with inflammatory low back pain. The symptoms are characterized by an insidious onset over several months, morning stiffness, and exacerbation of pain by rest. The evolution of AS is usually independent of the bowel disease, and more often runs a more benign clinical course than idiopathic AS[11].

ISOLATED SACROILIITIS

Isolated sacroiliitis may occur in association with IBD, without evidence of progressive spinal disease. Although some patients complain of low back pain, in many cases, they may be asymptomatic, therefore, prevalence is dependent largely upon the investigation. Radiographic surveys suggest a prevalence of 18%[7], but higher prevalence is estimated by means of scintigraphy, with radioisotope uptake found in up to 52% of patients with CD and 42% with UC[12]. However, given the large degree of inter- and intra-observer error, and the lack of follow-up information about possible progression to AS, the significance of these findings is unclear. In the majority of patients, it seems a non-progressive condition. In clinical practice, diagnosis is usually made by plain abdominal radiography, but more often it is detected incidentally.

PERIPHERAL ARTHRITIS

Articular manifestations have been reported in association with IBD for many years, but only in the late 1950s was arthritis proven to be inflammatory, and quite distinct from classical rheumatoid arthritis (RA), to be seronegative. Articular manifestations occur in 5%-20% of IBD patients, and more recently, a new classification proposes two distinct clinical forms with specific genetic associations[13].

Type I peripheral arthropathy, defined as acute, self-limiting inflammation that affects fewer than five joint, and is associated with IBD relapse and the presence of other extra-intestinal manifestations. More often, large weight-bearing joints are affected, particularly knees, wrists and ankles. The median duration of illness is 5 wk, however, 10%-20% of patients will develop persistent symptoms. One third of patients will report one episode over time.

Type Ⅱ arthritis is a symmetrical, seronegative, small-joint arthropathy, unrelated to disease activity. The onset may occur at any time during the course of IBD or before, and in CD, it is reported more commonly in colonic disease. It usually runs a more chronic course with a median duration of 3 years. The small joints of the hands are more commonly affected.

ROLE OF INTESTINAL INFLAMMATION

The role of intestinal inflammation and luminal factors in SpA is an area of debate. In a large series of ileocolonoscopy examinations with biopsies in patients with SpA[14,15], inflammatory lesions were found in two thirds of patients, with a similar proportion in those with AS and RA. Moreover, the clinical course of articular disease was independent of the presence of gut inflammation, and a minority of patients (3.7%) developed overt IBD. In another study[16], 123 patients with SpA at initial endoscopy were reassessed clinically, and follow-up endoscopy performed in 49 patients. Articular remission rates were independent of initial gut inflammation and associated with endoscopic and histological remission. In addition, initial chronic gut inflammation implies a high risk of evolution of AS. Nevertheless, a potential confounding factor of these studies is that most patients are treated with non-steroidal anti-inflammatory drugs (NSAIDs) which may promote enteropathy that is hardly distinguishable from primary inflammatory lesions of the gut. Furthermore, in a follow-up study[17] of some patients still taking NSAIDs, no inflammatory gut lesions were found in those in clinical remission from articular symptoms, while in contrast, half of the patients with articular inflammation had persistent inflammatory gut lesions, and one quarter developed overt IBD. This suggests that intestinal inflammation is important in idiopathic as well IBD-associated AS, possibly by determining an increased antigenic load across the inflamed gut mucosa.

Post-enteric reactive arthritis is associated with Gram-negative enterobacteria such as *Salmonella, Escherichia coli, Yersinia* and *Campylobacter*; in these circumstances, bacterial antigens have been isolated in the affected joints. These conditions are clinically very similar to type Ⅰ IBD arthritis, and it therefore seems likely that bacterial antigens may be important in the initiation of the inflammatory process. Furthermore, stronger support on the role of bacteria in initiating arthritis in the presence of gut inflammation has been gained from a study of HLA-B*27 transgenic animal models. These knock-out animals spontaneously develop colitis and axial and peripheral arthritis when reared under normal conditions[18,19]. Interestingly, when they are kept in a germ-free environment, gut and joint inflammation is prevented[19]. Furthermore, different bacteria induce gut and joint inflammation with different efficiency, with *Bacteroides vulgatus* being the most efficient and *E. coli* is ineffective[20]. Thus, it appears likely that similarly to IBD, bacteria are important in the pathogenesis of SpA, with an interaction with the immune system. The link between bacteria, gut/joint inflammation and the immune response, might result from a (common) genetic predisposition.

GENETIC PREDISPOSITION TO SPONDYLOARTHROPATHY

Idiopathic AS is strongly associated with possession of HLA-B*27, with a 94% prevalence in northern European patients, compared with 10% of healthy controls[21,22]. This association is considerably weaker in IBD-associated AS, ranging between 50% and 80% of patients[23-25]. Conversely, 50% of HLA-B*27-positive IBD patients have AS, compared with 1%-10% of B*27-positive individuals in the general population. Putative mechanisms include B*27 presenting peptide from luminal bacteria, or self proteins causing an inflammatory response, or peptides being presented to the immune system by other antigens such as HLA-DR. Central to these theories is the concept of a triggering bacterial antigen, although evidence is lacking. Patients with isolated sacroiliitis are less likely to be HLA-B*27-positive, and HLA-B*27 seems to be a marker of progressive axial disease rather than sacroiliitis, but long-term studies are needed. Other genes in the HLA region have also been implicated including HLA-DR1, TAP and LMP, but none of these findings have been conclusive. Moreover, subsequent studies have demonstrated other HLA associations in IBD arthritis. The association with HLA-DRB1*0103 is solely with type Ⅰ arthritis, which is also associated with HLA-B*27, whereas type Ⅱ is associated with B*44 and MICA[13,26] (Table 1).

A number of studies have demonstrated a striking overlap within patients and family members with rheumatological, dermatological and gastrointestinal diseases. The susceptibility genes of these disorders appear to overlap with each other[27]. In a study that explored the prevalence of secondary disorders in 3287 AS individuals[28], the sub-group of patients with IBD-AS had higher prevalence of iritis (OR = 1.4) or psoriasis (OR = 1.9) than the controls. Moreover, patients with multiple disorders predicted the highest prevalence of co-existing disease (i.e. psoriasis, IBD, iritis, or AS) within family members. These data suggest that susceptibility factors are additive or have a synergistic effect on each other, thus pointing to a shared gene hypothesis.

Table 1 HLA associations in IBD and IBD-associated arthropathy[11]

	HLA antigens investigated	Percentage in IBD + arthropathy affected patients	Percentage in IBD patients without arthropathy
AS	HLA-B*27	60	7
Type Ⅰ arthritis	HLA-B*27	26	7
	HLA-B*35	33	15
	HLA-DRB1*0103	35	3
Type Ⅱ arthritis	HLA-B*44	62	31

GENETIC PREDISPOSITION TO IBD

IBD is widely believed to originate from a dysregulated immune response to luminal bacteria in a genetically susceptible host[2]. The inheritance model is non-Mendelian but complex-polygenic, with several genes involved together with environmental factors. Of the other environmental factors thought to have an impact on disease susceptibility, only smoking and appendectomy have a substantive evidence base[29]. Concordance data in twins (36% for CD and 16% for UC, 4% for both CD and UC for monozygotic and dizygotic twins, respectively) and multiplex IBD families (relative risk to first-degree relative of proband up to 35 for CD and 15 for UC) have provided strong epidemiological evidence for a genetic contribution to IBD susceptibility[30]. These observations have led to the development of genetic investigations with two broad strategies: one has investigated candidate genes, and the other has used hypothesis-free methods of genome-wide scanning.

Overall, candidate gene strategies have not proved to be particularly fruitful in IBD, but one success has been the identification of an association between the major histocompatibility complex (MHC) region and UC susceptibility, initially in Japanese subjects[31] and later in Europeans[32]. The DRB1*0103 allele has been implicated in both severe UC and extra-intestinal (mainly articular) manifestations of IBD. Subsequently, 11 genome-wide scans by non-parametric linkage analysis were performed in the 1990s, and several susceptibility loci were identified in approximately half of the chromosomes[33].

The *NOD2* gene on chromosome 16q12 was the first susceptibility gene for CD to be identified successfully. It was detected by parallel strategies of positional cloning within a region of linkage and positional candidate gene investigation[34,35], and the finding was subsequently widely replicated in Caucasian, but not in Asian populations. NOD2 encodes an intracellular receptor that is expressed predominantly in monocytes and Paneth cells[33]. This has been implicated in the innate immune response to muramyldipeptide (MDP), a component of peptidoglycan

in bacterial cell walls. The CD-associated variants that are clustered in the C-terminal leucine-rich repeat part of the NOD2 protein significantly diminish responsiveness to MDP. On exposure to MDP, oligomerized NOD2 recruits RIP2 (the serine-threonine kinase RICK) and subsequently activates nuclear factor-κB transcription factor *via* NEMO ubiquitination and IκB degradation. This leads to production of cytokines and cryptidins/defensins, hence facilitating clearance of bacteria. In addition, there is evidence of cross-talk between NOD2 and toll-like receptor pathways. However, the precise mechanism by which NOD2 mutations lead to increased intestinal inflammation is unknown, but it may be that reduced ability to clear bacteria by innate immune mechanisms leads to dysregulation of adaptive immune pathways.

Following fine mapping of the IBD5 linkage region on chromosome 5q31, consistent evidence for association between CD and a haplotype of markers spanning 250 kb has been observed. More recently, the same locus has been associated also with UC[36]. However, because of the strong linkage disequilibrium (LD) across this region, it has been very difficult to identify the causal variant. Peltekova *et al*[37] have reported a two-locus risk haplotype in the region of the organic cation transporter (OCTN) genes and suggested that this accounts for the association. The two-locus haplotype comprises L503F (1672 C-T, missense substitution) and G207C (transversion) in the *SLC22A4* (OCTN1) and *SLC22A5* (OCTN2) genes, respectively. However, there remains significant debate as to whether these transcripts are truly implicated by the genetic evidence, and a number of other immunoactive candidates remain in the frame, including interferon regulatory factor 1 (IRF1) and a number of important cytokine genes (IL-3, IL-4, IL-5 and IL-13) located within the 250-kb risk haplotype.

Progress from the Human Genome Project and HapMap Project, combined with markedly decreasing genotyping costs, has made possible the performance of adequately powered genome-wide association studies (GWAs) in complex genetic disorders such as IBD. Several GWAs[38] and a meta-analysis[39] have already been performed in CD and more recently in UC[40] and pediatric IBD[41]. Genome scans have identified 11 susceptibility genes and loci and highlighted a number of new, previously unsuspected pathways as playing an important role in IBD pathogenesis, including the IL23 pathway in IBD overall and specific aspects of innate immunity (particularly the autophagy genes *ATG16L1* and *IRGM*) in CD. As expected, a number of genes such as IL23r, IL12B, MHC, STAT3, IBD5, MST1, PTPN2, NKX2-3 seem to be shared in the predisposition to both UC and CD (Table 2).

Table 2 Candidates genes and loci in CD and UC

	Genes and loci	Associated with CD	Associated with UC
1p31	IL23R	Yes	Yes
1q21	ECM1	-	Yes
2q37	ATG16L1	Yes	No
3p21	Several included MST1-BSN	Yes	Yes
5p13	Intergenic, PTGR4	Yes	No
5q31	Several included SLC22A5	Yes	Yes
5q33	IRGM	Yes	No
5q33	IL12B	Yes	Yes
6q21	HLA-DQ/DR	Yes	Yes
10q21	ZNF365	Yes	Yes
10q24	NKX2-3	Yes	Yes
16q12	NOD2	Yes	No
17q21	Several included STAT3	Yes	Yes
18p11	PTPN2	Yes	Yes

CD: Crohn's disease; UC: Ulcerative colitis.

COMMON GENETIC LINK BETWEEN IBD AND SPONDYLOARTHROPATHY

IBD and AS show familial clustering and may coexist in a patient. More intriguingly, healthy first-degree relatives of patients with AS (21%-60%) and CD (10%-54%) have increased permeability of the small intestine[42-44]. These changes might be a consequence of subclinical intestinal inflammation, and conform to the profile of an additive trait in both conditions[45,46]. Evidence from studies of twins and other first-degree relatives suggests that the genetic basis is somewhat stronger for IBD than for AS[47-50].

In the pre-GWA era, studies investigating a possible common genetic background in IBD and articular involvement were concentrated mainly on MHC and NOD2. It has been recognized that genes in the HLA region have a greater role for modifying IBD phenotype than in determining overall disease susceptibility. Within UC clinical subgroups, the uncommon DRB1*0103 allele is associated with both extensive and severe disease, with an early need for surgery[51]. Interestingly, the same allele has also been associated with CD colitis, thus suggesting the molecular basis of a colonic IBD phenotype. Furthermore, type I pauciarticular large-joint arthritis is also associated with this allele and other class I alleles (B*27 and B*35) in LD. However, the low frequency of this allele suggests that this association is unlikely to be clinically useful in predicting disease course. In addition, an increased prevalence of extraintestinal manifestations has been reported previously in colonic IBD, increasing the possibility that the association between DRB1*0103 and articular manifestations may merely mirror the association with colonic disease. Finally, recent evidence from GWA scans has at least refined the signal to the 400-kb haplotype block that contains DRB1*0103, and shows that this locus is common to UC and the colonic (but not small bowel) sub-phenotype of CD[52].

Crane *et al*[53] have investigated the hypothesis that the three major variants of the NOD2 gene are involved in AS. A case-controlled study was performed in 229 AS, 197 IBD-associated AS (78 with CD and 119 with UC), and 229 ethnically matched healthy controls. The Gly908Arg variant was associated with UC-associated AS (OR = 4.6, 95% CI = 1.3-16, $P = 0.016$), with a similar non-significant trend in CD-associated AS (OR = 3.9, 95% CI = 0.8-18, $P = 0.08$). In contrast, no association was found between

NOD2 variants and primary AS, or other variants with UC- or CD-associated AS. Moreover, carriage of the Pro268Ser variant was associated with greater disease activity. Similarly, in subsequent studies, variants of NOD2 do not appear to confer susceptibility to AS[54], but rather identify a subgroup of patient with CD-associated AS[55].

Recently, the Wellcome Trust Case Control Consortium and the Australo-Anglo-American Spondylitis Consortium have published the first association scan with 14 436 non-synonymous single nucleotide polymorphisms (SNPs) in 922 independent cases of AS, together with autoimmune thyroid disease, multiple sclerosis and breast cancer, against a common control dataset of 1500 healthy individuals[56]. The strongest association in AS was observed in the MHC region, centered around the HLA-B genes, but the association of $P < 10^{-20}$ was observed across about 1.5 Mb, probably reflecting the strong effect of HLA-B27, even over distant SNPs with modest LD. To validate less strong signals obtained at the first scan, a further 471 independent AS cases and 625 new controls were genotyped with additional SNPs. In the combined data set, a strong association ($P = 1.2 \times 10^{-8}$ to 3.4×10^{-10}) with SNPs of the ARTS1 gene (OR = 1.4) and IL23R gene (peak P value of 7.5×10^{-9} at the rs11209032 with an OR of 1.3) was found. The association with IL23R remained strong when considering individuals with AS not having IBD (n = 1066). These genes both represent excellent biological candidates, but more importantly, IL23R has been documented recently in CD and psoriasis[57], which suggests that this gene is a common susceptibility factor for the major seronegative diseases, at least partially explaining their co-occurrence. IL23R is a key factor in the regulation of a newly defined effector T-cell subset, T_H17 cells[33]. They express high levels of the cytokine IL-17 in response to stimulation, in addition to IL-1, IL-6, tumor necrosis factor α, IL-22 and IL-25. In animal models, blocking IL-23 reduces inflammation, which suggests that IL23R variants associated with disease are pro-inflammatory. No functional studies of IL23R variants are available to date, however a promising treatment of CD has been reported with anti-IL-12p40 antibodies, which block IL-12 and IL-23, as these cytokines share the p40 subunit[58].

A different perspective was employed in a study that explored the possible common genetic background of IBD and AS through a genealogical evaluation in Iceland[59]. Icelanders are relatively homogeneous with respect to the environment, cultural aspects and genetic factors; moreover, extensive genealogical records and diseases registries are available. By investigating the genealogical database and registry of subjects with AS (n = 205) and IBD (n = 1352), the risk ratios for relatives for each disease and the cross-risk ratios (AS *vs* IBD and *vice versa*) were estimated. First-, second- and third-degree relatives of patients with AS, had risk ratios of 94, 25 and 3.5, respectively, of developing AS (each $P < 0.0005$), while first-, second- and third-degree relatives of patients with IBD had risk ratios of 4.4, 2.2 and 1.4, respectively (each $P < 0.0001$). More intriguingly, the cross-risk ratio was 3.0 and 2.1 ($P < 0.0001$), respectively, in first- and second-degree relatives, with a comparable effect for UC and CD. This elevated cross-risk ratio between IBD and AS strongly suggests that there is a genetic component shared by these complex diseases, and should stimulate further molecular and functional studies.

CONCLUSION

The clinical association between spondyloarthropathy and IBD is well-established, with studies indicating that as many as 10%-15% of cases of IBD are complicated by AS or other forms of SpA[60]. Ileal inflammation that resembles IBD has been reported in up to two thirds of cases of SpA, and it has been suggested that the presence of ileitis is associated with the chronicity of articular complications. Moreover, evidence that there is familial clustering of IBD and AS, that both conditions may coexist in patients, that there is an increased risk ratio among first- and second-degree relatives of affected AS or IBD patients and finally, that there is an increased cross-risk ratios between AS and IBD, confirm the existence of a shared genetic predisposition. So far, IL23R is the only identified susceptibility gene shared by IBD and AS. Functional studies are still needed to better understand its functional role, but it is hoped that treatment that targets this pathway may prove effective in both disorders.

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TOPIC HIGHLIGHT

Walter Fries, MD, *Series Editor*

Non-invasive investigation in patients with inflammatory joint disease

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Abstract

Gut inflammation can occur in 30%-60% of patients with spondyloarthropathies. However, the presence of such gut inflammation is underestimated, only 27% of patients with histological evidence of gut inflammation have intestinal symptoms, but subclinical gut inflammation is documented in two-thirds of patients with inflammatory joint disease. There are common genetic and immunological mechanisms behind concomitant inflammation in the joints and intestinal tract. A number of blood tests, e.g. erythrocyte sedimentation rate, orosomucoid, C-reactive protein, and white cell and platelet counts, are probably the most commonly used laboratory markers of inflammatory disease, however, these tests are difficult to interpret in arthropathies associated with gut inflammation, since any increases in their blood levels might be attributable to either the joint disease or to gut inflammation. Consequently, it would be useful to have a marker capable of separately identifying gut inflammation. Fecal proteins, which are indirect markers of neutrophil migration in the gut wall, and intestinal permeability, seem to be ideal for monitoring intestinal inflammation: they are easy to measure non-invasively and are specific for intestinal disease in the absence of gastrointestinal infections. Alongside the traditional markers for characterizing intestinal inflammation, there are also antibodies, in all probability generated by the immune response to microbial antigens and auto-antigens, which have proved useful in establishing the diagnosis and assessing the severity of the condition, as well as the prognosis and the risk of complications. In short, non-invasive investigations on the gut in patients with rheumatic disease may be useful in clinical practice for a preliminary assessment of patients with suspected intestinal disease.

Key words: Biochemical markers; Fecal markers; Inflammatory bowel disease; Intestinal permeability; Serological markers; Spondyloarthropathies

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INTRODUCTION

Gut lesions are relatively common in patients with rheumatic disease and approximately 30%-60% patients with spondyloarthropathies have occult intestinal inflammation, which may be related to their ingestion of non-steroidal anti-inflammatory drugs or associated with their rheumatic disease.

Spondyloarthropathies form a group of chronic autoimmune disorders of the joints which include ankylosing spondylitis, reactive arthritis, psoriatic arthritis, arthritis associated with inflammatory bowel disease (IBD), and undifferentiated spondyloarthropathies. The prevalence of gut inflammation in ankylosing spondylitis is higher among patients with associated peripheral arthritis than in those with axial involvement alone[1].

Gut inflammation has also been recorded in other spondyloarthropathies. In juvenile chronic arthritis, for instance, when colonoscopy with biopsies of the colonic mucosa and terminal ileum was performed in 12 patients less than 16 years of age, inflammation was observed in 9 patients (75%); gut inflammation could have a

role in the pathogenesis of the disease and persistent synovitis[2].

Two histological types of gut inflammation can be distinguished in spondyloarthropathies, i.e. acute and chronic, based on their morphological characteristics, not on the time of onset or duration of the disease[3]. The acute type resembles acute bacterial enterocolitis, with a well-preserved mucosal architecture. The chronic type resembles chronic ileocolitis and is generally indistinguishable from Crohn's disease, with a clearly disrupted mucosal architecture. While acute lesions are mainly seen in patients with reactive arthritis, chronic lesions are more prevalent in undifferentiated spondyloarthropathies and ankylosing spondylitis[4].

In a prospective study on 123 patients with spondyloarthropathies who initially underwent endoscopy, intestinal evolution was evaluated by ileocolonoscopy and an evolution to IBD was recorded in 7% of these patients[5].

Despite the high frequency of gut lesions in patients with joint diseases, only a few patients are symptomatic. In a series described by Cuvelier *et al*[3], only 27% of patients with histological gut inflammation had intestinal symptoms.

Non-invasive laboratory tests might therefore help to identify rheumatic disease patients with gastrointestinal symptoms who need further investigation. It would be helpful to have inexpensive and manageable tests to facilitate this selection.

GUT-RELATED GENETIC POLYMORPHISMS

There is clinical evidence of a correlation between gut and joint inflammation and the gut could have an important pathogenic role. Remission of joint inflammation has been associated with the disappearance of gut inflammation, and remission of persistent joint inflammation with the disappearance of persistent gut inflammation[4]. Ankylosing spondylitis affects 3%-10% of patients with IBD and is thought to have a genetic origin in these patients that differs from that of "classic" ankylosing spondylitis: while 90% of patients with "classic" ankylosing spondylitis have the human leukocyte antigen B27 phenotype, its prevalence drops to 30% in patients with ankylosing spondylitis associated with Crohn's disease. Polymorphisms of the CARD15 gene may act as a genetic trigger because 78% of patients with Crohn's disease and symptomatic or asymptomatic sacroileitis carry at least one mutation, as opposed to 48% of control patients with Crohn's disease alone[6]. Laukens *et al* confirmed a similar association, finding CARD15 variants in 42% of patients with spondyloarthropathy and asymptomatic gut inflammation, compared with 7% of patients with normal gut histology[7].

In a previous study, the frequency of HLA-Bw62 was found to be very high in patients with reactive arthritis and in those with active ankylosing spondylitis and Crohn-like lesions on gut biopsy[8].

BLOOD MARKERS

A number of proteins are up- or down-regulated in the acute phase of inflammation, and gut inflammation is associated with an acute-phase reaction and the migration of leucocytes to the gut lumen. Several blood tests are used to detect inflammation, however, these tests are unable to discriminate between inflamed joints and inflamed gut[9]. In a study on children with spondyloarthropathies, erythrocyte sedimentation rate (ESR) showed 63% sensitivity and 44% specificity in detecting gut inflammation[10]. Serum levels of human cartilage glycoprotein 39 (also called YKL-40) were recently found to be higher than normal in patients with IBD. More than 60% of Crohn's disease patients with extraintestinal manifestations have high serum YKL-40, as opposed to only 3% of ulcerative colitis patients[11]. We found significantly higher serum levels of YKL-40 in IBD patients with arthropathies than in those without arthropathies or controls ($P < 0.001$ and $P < 0.01$, respectively). The level of this protein also correlates with the number of joints involved, suggesting that this substance could be used as a disease activity marker in arthritis associated with IBD[12].

FECAL MARKERS

As serum markers may increase in various conditions, fecal markers might be more specific for gut inflammation. Barabino *et al*[10] compared a number of non-invasive tests for diagnosing intestinal inflammation in children with spondyloarthropathies. Forty-two children with IBD or rheumatologic manifestations associated with gastrointestinal symptoms were investigated by 99mTechnetium-HMPAO labeled white cell scanning, abdominal ultrasound, ESR, fecal occult blood and fecal alpha 1-antitrypsin tests. 99mTechnetium-HMPAO labeled white cell scanning was shown to be the most sensitive (85%) and specific (100%) in detecting gut inflammation. White cell scanning combined with the measurement of fecal excretion of labeled white cells was able to quantify inflammation accurately in an additional study: following intravenous administration of 111In-labelled leukocytes, fecal 111In granulocyte excretion correlated significantly with Crohn's disease activity index ($P < 0.001$), C-reactive protein (CRP) ($P < 0.001$) and ESR ($P < 0.001$)[13]. This technique is expensive and time-consuming, however, and involves the use of radiation. Moreover, leukocytes do not survive for long periods in feces due to bacterial degradation[14]. As an alternative, fecal leukocytes can be seen under the microscope, but again, such an evaluation is not practicable because it has to be carried out on fresh stools. Some leukocyte proteins, such as lactoferrin and calprotectin, are more durable and can be used as surrogate markers of the presence of leukocytes in stools. Fecal calprotectin levels have been shown to correlate with intestinal inflammation, as assessed by 111Indium-labeled leukocyte studies on 4-d-old fecal samples and the correlation was maintained, even when

a single stool specimen was examined[15,16].

A number of neutrophil-derived proteins have been studied in stools, including fecal calprotectin, lactoferrin, lysozyme, elastase and myeloperoxidase[17,18]. Experience with the analysis of fecal proteins has been gained mainly with calprotectin and lactoferrin. Calprotectin represents 60% of the cytosolic proteins in granulocytes, and is released from cells during cell activation or death, while lactoferrin is a component of the granules in the neutrophilic granulocytes, so their presence in feces is presumably directly proportional to neutrophil migration in the gut lumen[19,20]. Both calprotectin and lactoferrin are stable in stools for more than 7 d at room temperature[19,20].

Determining intestinal inflammation by means of fecal markers is of considerable interest to clinicians in various settings, e.g. to discriminate between patients with organic and functional processes, to monitor disease activity and response to treatment, and to predict relapses in IBD. Both calprotectin and lactoferrin have been found to correlate with intestinal inflammation in studies on patients undergoing colonoscopy for gastrointestinal symptoms or surveillance[21,22].

In a recent study, calprotectin and lactoferrin appeared to be equally recommendable as inflammatory disease markers in patients with lower gastrointestinal symptoms and both reflected inflammatory activity in IBD[23].

Fecal calprotectin and lactoferrin are equally useful in assessing disease activity: calprotectin correlated with endoscopic findings, lactoferrin with histology[23,24].

Fecal calprotectin also proved useful in predicting relapses in patients in clinical remission, probably reflecting subclinical activity. Tibble *et al*[25] found that fecal calprotectin levels greater than 50 μg/g were a sensitive and specific predictor of relapse in the short term in both ulcerative colitis and Crohn's disease (with 90% sensitivity and 83% specificity). More recently, Costa *et al*[26] found that ulcerative colitis patients with fecal calprotectin levels higher than 150 μg/g had a 14-fold relapse risk, while Crohn's disease patients had only a two-fold risk of relapse, which was not statistically significant. D'Incà *et al*[27] observed that calprotectin levels beyond 130 mg/kg correlated significantly with the probability of relapse in ulcerative colitis patients ($P = 0.000$) and colonic Crohn's disease patients ($P = 0.02$), but not in patients with ileal or ileocolonic disease.

INTESTINAL PERMEABILITY

Permeability refers to the property of a membrane that enables a solute to pass through it by unmediated diffusion due to the membrane's structure, the physical and chemical properties of the solute, and its interaction with the medium or solvent. Intestinal permeability is assessed non-invasively *in vivo* by measuring the urinary excretion of orally administered hydrosoluble, non-toxic and non-degradable probes, e.g. lactulose/mannitol, lactulose/rhamnose, ^{51}Cr-EDTA/rhamnose, or D-xylose.

Bjarnason *et al* postulated that a greater intestinal permeability to toxic "non-absorbable" compounds might be responsible for some of the extraintestinal tissue damage common in alcoholic patients[28]. An altered intestinal permeability may also represent the primary defect in patients with arthropathy. An increased antigenic load could result from an altered intestinal permeability, since higher levels of antibodies to *Klebsiella pneumoniae* have been found in the serum of patients with ankylosing spondylitis, rheumatoid arthritis and IBD[29]. Morris *et al*[30] found that small intestinal permeability increased in patients with ankylosing spondylitis taking non-steroidal anti-inflammatory drugs, suggesting that the increased permeability was probably not a primary mucosal lesion, but caused by the medication. De Vos *et al*[31] found both acute and chronic inflammation at the macroscopic (30%) and histological (61%) level in the terminal ileum of patients who were seronegative for arthropathy and were not taking non-steroidal anti-inflammatory drugs. Chronic inflammation predominated in ankylosing spondylitis patients, resembling Crohn's disease in one third of patients. Mielants *et al*[32,33] observed a greater gut permeability in rheumatic patients irrespective of whether they were taking non-steroidal anti-inflammatory drugs, indicating that the disrupted permeability is disease-related.

Altered gut permeability was also seen in juvenile chronic arthritides, which are frequently associated with IBD, despite the use of non-steroidal anti-inflammatory drugs ($P = 0.210$), disease activity ($P = 0.24$) and type of disease ($P = 0.28$)[34].

The same findings have been reported in various intestinal conditions, such as celiac disease[35], IBD[36], infectious gastroenteritis[37], and food intolerance or allergy[38,39]. We had the opportunity to study 261 consecutive patients referred with chronic diarrhea and found that the intestinal permeability test and CRP levels were independent predictors of the final diagnosis of an underlying organic small bowel disease. The test correctly identified the presence of organic disease in 80% of patients[40].

The permeability test is used in Crohn's disease to monitor disease activity and as a predictor of relapse in quiescent Crohn's disease. In active Crohn's enteritis, 95% of patients have an increased intestinal permeability, while in Crohn's colitis this is true of about 50% of patients[41]. Studies in patients with Crohn's disease in remission have shown that an increased intestinal permeability can pinpoint those at significant risk of disease relapse within the next few months[42,43].

SEROLOGICAL MARKERS

Serological tests focus on several antibodies, the most widely used being perinuclear anti-neutrophil cytoplasmic antibodies (p-ANCA) and anti-Saccharomyces cerevisiae antibodies (ASCA). p-ANCA were first described in ulcerative colitis patients in 1990, but the exact epitope remains unknown[44,45]. ASCA are directed against the cellular wall of baking yeast.

The specificity of both markers is very high, but their sensitivity is rather low and these tests are consequently

not suitable for screening purposes[46]. Combining the two (p-ANCA and ASCA) may be helpful, however, in the differential diagnosis between ulcerative colitis and Crohn's disease the combination of ASCA+/p-ANCA- is characteristic of Crohn's disease, while ASCA-/p-ANCA+ is characteristic of ulcerative colitis, with a sensitivity that ranges from 30%-64%, a specificity beyond 90% and a positive predictive value between 77% and 96%[47-49]. ASCA positivity has been related to disease severity[50], the risk of having to undergo surgery[51], and an ileal and/or right colonic localization of the disease[48,52].

The recent finding that p-ANCA and ASCA can be found in 25%-30% of patients some years before any IBD is diagnosed has shed some light on its pathogenesis[53].

Two studies documented a higher prevalence of ASCA IgA positivity in ankylosing spondylitis[54,55], adding proof to the conviction that spondyloarthropathies and IBD are immunologically related. Additional serum biomarkers include antibodies against outer membrane porin C (Anti-OmpC), the *Pseudomonas fluorescens* bacterial sequence I2 (anti-I2), bacterial flagellin (antiCBir1) and the anti-glycan antibodies, i.e. anti-chitobioside IgA (ACCA), anti-laminaribioside IgG (ALCA) and anti-mannobioside (AMCA)[46,56-58]. Although the data from independent studies vary, combining more than one serological marker has been shown to add clinical value, particularly in predicting a complicated disease behavior, including strictures, fistulas and the need for surgery.

CONCLUSION

Patients with spondyloarthropathies often have inflammation in the gut, especially in the terminal ileum, although only 30% of the patients involved have clinical symptoms.

The frequency of gastrointestinal disease remains poorly understood and should be investigated in all patients with chronic spondyloarthropathy. The early diagnosis and treatment of gut inflammation may make it unnecessary to use drugs that can damage the intestinal mucosa[59-61].

Biochemical markers are useful in managing gut inflammation, representing a valuable aid in the diagnosis of inflammatory processes and the evaluation of their prognosis.

The ideal marker should be easy to test, repeatable and inexpensive. Currently used blood markers are non-specific and reflect both joint and intestinal inflammation. The fecal markers calprotectin and lactoferrin, and intestinal permeability are more promising tests, as they have a good specificity for intestinal disorders and are straightforward to perform. This is very important, particularly to pinpoint those patients without intestinal symptoms who need to be selected for further, more invasive investigations, and to avoid medication-related complications.

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TOPIC HIGHLIGHT

Walter Fries, MD, *Series Editor*

Combined therapeutic approach: Inflammatory bowel diseases and peripheral or axial arthritis

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Abstract

Inflammatory bowel diseases (IBDs), particularly Crohn's disease (CD) and ulcerative colitis (UC), are associated with a variety of extra-intestinal manifestations (EIMs). About 36% of IBD patients have at least one EIM, which most frequently affect the joints, skin, eyes and the biliary tract. The EIMs associated with IBD have a negative impact on patients with UC and CD, and the resolution of most of them parallels that of the active IBD in terms of timing and required therapy; however, the clinical course of EIMs such as axial arthritis, pyoderma gangrenosum, uveitis, and primary sclerosing cholangitis is independent of IBD activity. The peripheral and axial arthritis associated with IBD have traditionally been treated with simple analgesics, non-steroidal anti-inflammatory drugs, steroids, sulfasalazine, methotrexate, local steroid injections and physiotherapy, but the introduction of biological response modifiers such as tumor necrosis factor-α blockers, has led to further improvements.

Key words: Anti-tumor necrosis factor antagonists; Inflammatory bowel disease; Treatment; Arthropathies

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INTRODUCTION

Inflammatory bowel diseases (IBD), particularly Crohn's disease (CD) and ulcerative colitis (UC), are associated with a variety of extra-intestinal manifestations (EIMs)[1]. About 36% of IBD patients have at least one EIM[2], and these have a negative impact on UC and CD patients. The most frequent EIMs affect the joints, skin, eyes and the biliary tract[3], but others give rise to small bowel dysfunctions (cholelithiasis, nephrolithiasis and obstructive uropathy) or are non-specific disorders (osteoporosis, hepatobiliary disease and amyloidosis)[1,2]. Although the association between EIMs and IBD has long been recognized, the underlying pathogenetic factors remain unclear. The reported incidence of peripheral and axial arthropathies ranges from 4% to 23%[4,5].

The Oxford group distinguished two types of peripheral arthropathy on the basis of their articular involvement[4]: (1) Type I is a large joint pauci-articular arthropathy that mainly affects the ankles, knees, hips, wrists, elbows and shoulders; is usually acute and self-limiting; occurs at times of IBD activity; and leaves no permanent joint damage. (2) Type II is a polyarticular arthropathy that mainly affects the small joints of both hands symmetrically; is characterised by pain that usually persists for months or years; and is largely independent of IBD activity.

Axial arthritis includes sacroiliitis and ankylosing spondylitis (AS)[5]: (1) Sacroiilitis may be asymptomatic or symptomatic[6]; asymptomatic sacroiilitis is common and up to 50% of CD patients show abnormal radiographic findings; symptomatic sacroiliitis is characterised by pain in the pelvis after rest, which improves with movement, and discomfort in the sacroiliac joints during bilateral pressure on the pelvic brim. (2) AS is characterised by persistent low back pain beginning before the age

of 30 years, and its clinical diagnosis is supported by characteristic radiological changes for which magnetic resonance imaging is the diagnostic tool of choice[7,8]. Although HLA B-27 is over-represented in IBD-related axial arthritis, it is of no diagnostic value[9].

THERAPEUTIC APPROACHES TO IBD AND ARTHROPATHIES

The treatment of IBD-associated arthropathies is almost entirely based on extrapolations from other forms of arthritis.

In the case of type I peripheral arthritis, treatment should concentrate on the active disease and include steroids, immunomodulators and anti-tumor necrosis factor-α (TNF-α)[10-12]; however, all forms of IBD-related arthritis seem to be treated with sulfasalazine (SSZ) despite the lack of supportive evidence[13]. The symptoms can be relieved using simple analgesics, rest and physiotherapy. Non-steroidal anti-inflammatory drugs may aggravate the underlying colitis[14], but the findings of a randomised study regarding the safety of celecoxib[15] indicate that its short-term use (< 2 wk) does not exacerbate colitis. A local steroid injection into the affected joints provides rapid but only temporary relief.

The treatment of AS should include intensive physiotherapy, together with the administration of disease-modifying drugs such as SSZ and methotrexate. However, since TNF-α has been shown to play a key role in the pathogenesis of AS and IBD, the treatment of this manifestation has changed[16].

The advent of biological response modifiers such as TNF-α blockers has improved the treatment of IBD and its associated peripheral and axial arthritides, and their safety and efficacy have been clearly established in the case of AS-related peripheral arthritis. IBD patients failing on immunomodulation therapy used to be recommended surgery, but are now treated with biological agents.

Table 1 lists the currently used treatments for IBD-related arthropathies.

Table 1 Therapy of spondyloarthropathies

Therapy	
Standard initial therapy	NSAIDs and anti-COX-2
	Physical activity
	Local steroids
Second line therapy	Sulfasalazine
	Methotrexate
	Gold
	Others (penicillamine, *etc*)
Biological therapies (TNFα-blockers)	Infliximab
	Etanercept
	Onercept
	Adalimumab
	Thalidomide

Anti-TNF antagonists

Infliximab is a chimeric IgG1 monoclonal antibody to TNF and represents a significant advance in the treatment of IBD with or without associated arthropathies[17]. There are anecdotal accounts of infliximab rapidly improving peripheral arthritis in IBD patients. Ellman *et al*[18] have reported the findings of an open-label study in which four patients with treatment-refractory peripheral arthritis responded to treatment with infliximab 5 mg/kg and, subsequently, a large-scale prospective, open-label trial demonstrated an improvement in peripheral arthritis in IBD patients who had previously been refractory to corticosteroids, 6-mercaptopurine, azathioprine or methotrexate[19]. Another small open-label study documented an improvement in the arthralgias of seven out of 11 IBD patients after a single infusion of infliximab 5 mg/kg[20].

On the basis of the available data, it seems that most IBD patients with active intestinal inflammation and concurrent peripheral arthritis are likely to experience an improvement in their joint symptoms upon receiving infliximab.

Adalimumab is a subcutaneously self-administered fully human monoclonal antibody against TNF-α that is efficacious in inducing and maintaining remission in patients with moderate-to-severe CD[21], but there are no published data concerning its efficacy in patients with concomitant IBD and arthritis.

Infliximab, etanercept and adalimumab have all been found to have positive short- and long-term effects on disease signs and symptoms in AS patients[22,23]. Braun *et al*[24] analysed the data from nine trials of anti-TNF agents (seven placebo-controlled and two open-label studies) and found that the treatment is efficacious in treating AS and IBD, and that the onset and flare of IBD are infrequent events in AS patients receiving anti-TNF therapy. Infliximab (but not etanercept) largely prevents IBD and AS activity but more data are required in the case of adalimumab.

The efficacy of adalimumab in the treatment of AS is mainly supported by the findings of the recent multicentre, randomized, double-blind and placebo-controlled trial conducted by van der Heijde *et al*[25] who observed that the response of most of the patients treated with adalimumab was better than that observed in the patients treated with placebo.

No published studies have yet addressed the effect of switching from infliximab to adalimumab in patients with CD-related spondyloarthropathy. We have recently evaluated the clinical response to adalimumab of 19 CD patients with associated spondyloarthritis who discontinued infliximab because of intolerance or loss of efficacy, and found that it successfully controlled both articular and intestinal disease activity[26].

In conclusion, about 36% of IBD patients have at least one EIM, especially articular involvement, and the introduction of anti-TNF therapy has improved the treatment of both. In particular, the subgroup of Crohn's disease patients with arthritic problems could be the one in which anti-TNF agents are most indicated.

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TOPIC HIGHLIGHT

Walter Fries, MD, *Series Editor*

Common immunologic mechanisms in inflammatory bowel disease and spondylarthropathies

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Abstract

Spondyloarthropathies (SpA) are commonly observed extra-intestinal manifestations of both Crohn's disease (CD) and ulcerative colitis (UC), the two major forms of inflammatory bowel diseases (IBD). However, the immunological link between these two clinical entities is still poorly understood. Several lines of evidence indicate that SpA may originate from the relocation to the joints of the immune process primarily induced in the gut. The transfer of the intestinal inflammatory process into the joints implicates that immune cells activated in the gut-draining lymph nodes can localize, at a certain point of the intestinal disease, either into the gut or into the joints. This is indicated by the overlapping expression of adhesion molecules observed on the surface of intestinal and synovial endothelial cells during inflammation. Moreover bacterial antigens and HLA-B27 expression may be implicated in the reactivation of T cells at the articular level. Finally, accumulating evidence indicates that a T helper 17 cell-mediated immune response may contribute to IBD and IBD-related SpA with a crucial role played by tumor necrosis factor-α in CD and to a lesser extent in UC.

Key words: Cell adhesion molecules; Antigens; Th17; Helper T-cells; Tumor necrosis factor-α

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INTRODUCTION

The term spondyloarthropathy (SpA) indicates a group of related diseases, including ankylosing spondylitis (AS), reactive and psoriatic arthritis (ReA and PsA), and undifferentiated spondyloarthritis (uSpA). All these forms of SpA share common clinical features which are sacroiliitis, inflammatory low back pain and oligoarticular asymmetric synovitis. SpA is a frequently observed extraintestinal manifestation in Crohn's disease (CD) and ulcerative colitis (UC), the two major forms of inflammatory bowel diseases (IBD). Indeed, the prevalence of SpA associated with CD and UC was 45.7% and 9.9%, respectively in a recent series[1]. Moreover a subtle gut inflammation is present in 25%-75% of patients with documented SpA[2], depending on the subtype, and among these, 6%-13% may evolve to overt IBD, suggesting common pathogenetic mechanisms between these two clinical entities[3-5].

The observation that SpA may occur during IBD has led to the hypothesis that IBD-related SpA originates from extraintestinal spreading of the immunologic process originating in the gut. Results from several studies suggest that the activation of the intestinal immune system may indeed lead, in certain conditions, to the generation of T cell clones which leave the gut compartment to home into the joints. These T cell clones would be able to replicate, in this site, the inflammatory process observed in the gut. However, to render this model plausible some conditions need to be met. Indeed the idea that joint inflammation is driven by T cell clones originating in the gut implies that these cells are able to leave the gut and to transfer into the joints. Secondly, gut-derived T cells need to encounter in the joint environment an adequate antigenic stimulus to

allow the reactivation of these cells. Finally, the immune response shaped in the gut must be responsible for the inflammation-related tissue damage observed in SpA.

FROM THE GUT TO THE JOINT: THE T CELL HOMING

A critical point for transfer of the inflammatory process from the gut to the joints is the possibility of redirecting the tissue-specific homing of inflammatory cells, mainly T cells, into the synovial compartment. In IBD, the abnormal reactivity of T cells against harmless antigens expressed by the commensal flora is thought to cause chronic intestinal inflammation[6,7]. In the gut-associated lymphoid tissue (i.e. Peyer's patches and lymphoid follicles) and mesenteric lymph nodes, professional antigen presenting cells (i.e. dendritic cells, DC) migrate from the intestinal lamina propria, prime naïve T cells which in turn differentiate into specialized T helper cells (e.g. Th1, Th2, Th17), thus acquiring the capacity to sustain a specific immune response. The profound changes observed in differentiated T cells in the secondary lymphoid organs include the expression of cell surface adhesion molecules and chemokine receptors which are responsible for the gut-specific T cell homing. Indeed, T cells activated in the Peyer's patches and mesenteric lymph nodes express the gut-addressing integrin $\alpha4/\beta7$ and the chemokine receptor CCR9[8]. Once activated, these cells reach the bloodstream through the efferent lymphatics and the thoracic duct. In the gut mucosa, the interaction between $\alpha4/\beta7$ integrin and its ligand, the mucosal addressin cell adhesion molecule 1 (MadCAM-1) expressed on the venular endothelial sheet[9,10] causes the initial rolling and subsequent arrest of activated T cells. MadCAM-1 is normally expressed on the intestinal mucosa and its expression is further enhanced during inflammation[11]. Once arrested on the surface of the intestinal venules, activated T cells transmigrate through the endothelial layer and move into the lamina propria following the gradient formed by the CCR-9-specific ligand CCL-25[12,13]. Therefore, the specific interaction between $\alpha4/\beta7$ integrin with MadCAM-1 and CCR9 with CCL-25 is pivotal for T cell homing into the gut. However it is worth noting that other molecules mediate the cell-to-cell interaction in this process. For instance CD44, the very late antigen-4 (VLA-4, $\alpha4\beta1$) and the lymphocytes function associated antigen-1 (LFA-1, $\alpha L\beta2$) expressed by activated T cells play a role in the recruitment of T cells into the gut. Moreover the expression of the vascular activated peptide-1 (VAP-1), intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and different subgroups of selectins (i.e. P- and E-selectins) which bind P-selectin glycoprotein-1 (PSGL-1) on T cells, is enhanced in endothelial cells of the inflamed intestine[14]. However, these molecules are not gut specific and they seem to contribute marginally to the specificity of T cell homing into the intestine. Nevertheless, many lines of evidence indicate that these molecules may be involved in the homing of gut-activated

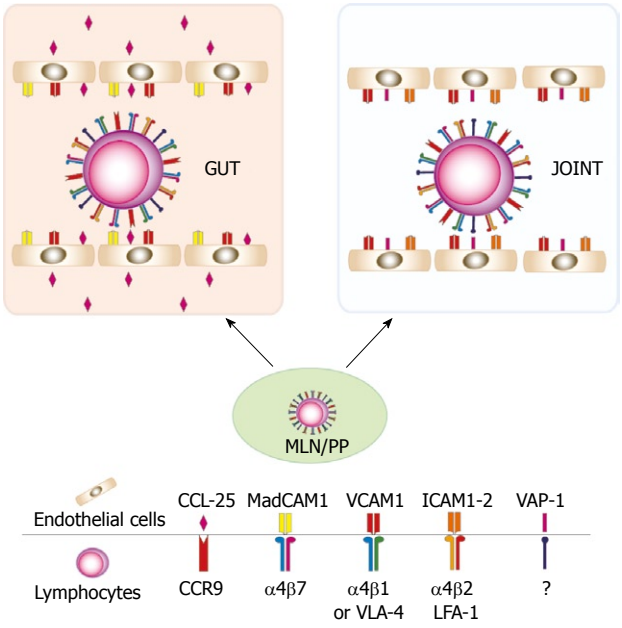

Figure 1 The heterogeneous expression of adhesion molecules allows T cells activated in the gut to home into the joints. CCR9: Chemokine receptor-9; CCL-25: Chemokine ligand-25; MadCAM1: Mucosal addressin cell-adhesion molecule-1; VCAM1: Vascular cell adhesion molecule-1; ICAM : Intracellular adhesion molecule; VLA-4: Very late antigen-4; LFA-1: Lymphocyte function associated antigen-1; VAP-1: Vascular adhesion protein-1.

T cells in other organs. Initial studies indicated that lamina propria lymphocytes (LPL) isolated from uninflamed gut were able to bind to uninflamed synovial vessels and that this interaction was critically mediated by CD44, VLA-4 and LFA-1[15]. In contrast, the adherence of lamina propria T cells to inflamed synovial vessels was dependent on VAP-1 and CD44[16]. When LPL were isolated from the inflamed gut of IBD patients, these cells bound more efficiently to synovial vessels than cells isolated from uninflamed gut[17]. This higher affinity could be explained by the observation that, in contrast with cells isolated from uninflamed gut, the binding of small, memory-like, T cells from IBD patients was, in addition to VLA-4, $\alpha4\beta7$-dependent, thus indicating a more varied use of adhesion molecules by IBD lamina propria T cells. By contrast, immunoblasts isolated from the lamina propria of IBD patients relied on CD44, LFA-1 and VAP-1 for their adhesion to synovial vessels, similar to immunoblasts from uninflamed gut.

Overall these data indicate that T cells primed in the gut-draining secondary lymphoid organs express a pattern of adhesion molecules that in part are responsible for the intestinal specific homing but that might, in particular conditions, mediate the entrance of activated T cells into extraintestinal compartments such as synovial tissue (Figure 1).

ROLE OF ANTIGEN MIMICRY AND HLA-B27 IN THE ANTIGENIC STIMULATION

As previously mentioned, intestinal commensal bacteria

are thought to sustain intestinal inflammation in IBD. Analogously, IBD-related SpA is thought to depend on the interaction between the host and the gut microbiota. This hypothesis is supported by the observation made in rat models of colitis and colitis-associated SpA. These rats, characterized by the over-expression of human HLA-B27, spontaneously develop intestinal inflammation and SpA. However, these animals did not develop inflammation when grown in germ-free conditions[18]. In contrast, both intestinal inflammation and SpA reappeared when they were transferred to a specific pathogen free environment[19,20], thus indicating that the intestinal and articular inflammatory processes depend on the presence of bacteria into the gut lumen.

Although many attempts to isolate living pathogens from the joint fluid during reactive arthritis failed, the presence of *Yersinia enterocolitica*-, *Salmonella enteritidis*- and *typhimurium*-, *Yersinia*- and *Shigella*-related antigens have been detected in the joints of these patients[21-23]. This observation suggests that the enteric antigens may be transported into the joints by monocytes. Accordingly, macrophages from the lamina propria of IBD patients were shown to adhere to the endothelial cells of synovial tissue[17]. It is therefore possible that recirculation of antigen-loaded macrophages may provide the antigenic stimulus necessary to sustain T cell activation and inflammation in the joints.

The crucial role of antigen stimulation in the pathogenesis of IBD-related SpA is also supported by the strong genetic association between SpA and the human leukocyte antigen (HLA) class I B-27 (HLA-B27). HLA-B27 was found in 75%-95% of patients affected by SpA[24,25] and in 25%-78% of IBD patients without SpA who developed this extraintestinal manifestation at a later stage of the disease[26,27]. Despite the strong genetic association, the pathogenetic role of HLA-B27 are still poorly understood. Activation of CD8+ T cells involved in the arthritic process by specific bacterial antigens exposed on HLA-B27 has been proposed[28,29]. Moreover, it has been shown that CD4+ T cells isolated from patients with reactive arthritis are activated by bacterial peptides presented in the context of HLA-B27[30]. These data draw a possible scenario in which activated CD4+ T cells migrate into the joints from the gut, in response to bacterial antigens presented by HLA-B27-expressing macrophages. Not necessarily in contrast with this hypothesis are data demonstrating the homology between HLA-B27 sequences and antigens derived from virus and enterobacteria. Indeed a certain level of antigen mimicry may contribute to the onset and/or maintenance of the inflammatory process initially induced by bacterial antigens. For instance, the nonapeptide, LRRYLENGK, derived from HLA-B27 (residues 168-176) shares the same sequence as peptides from enteric organisms (i.e. *Pseudomonas aeruginosa, Klebsiella nitrogenase, Escherichia coli, Bacillus megaterium, Salmonella typhimurium*). The same peptides originating from either bacteria or HLA-B27 endogenous turnover can be presented by HLA-B27, determining the activation of the same T cell repertoire[31]. Moreover, a dodecapeptide contained in the intracytoplasmic tail of HLA-B27 shows a strong homology with the sequence contained in the DNA primase of the arthritogenic bacteria *Chlamydia trachomatis*[32]. This fits well with the observation that high titers of antibodies anti-Saccharomyces cerevisiae (ASCA) and anti-neutrophils (pANCA) antibodies are present in IBD-related SpA[33]. Indeed pANCAs have been shown to cross-react with both neutrophil nuclear membrane and a *E. coli* proteins[34], while no self-antigens have been so far identified for ASCA. These data indicate that in the presence of HLA-B27 but also independently of this HLA antigen, an immune response initially evoked by a bacterial antigen may be further sustained by the cross-reactivity with self-antigens.

Recently, the process of folding and expression of the HLA-B27 heavy chain has received increasing attention as a potential mechanism involved in the pathogenesis of SpA. Under normal conditions, the peptide-loaded HLA class I heavy chain binds the β2-microglobulin (β2m). This assembling process takes place in the endoplasmic reticulum[35]. The folding process of the HLA-B27 heavy chain is slower than that of other HLA alleles thus leading to the generation of misfolded chains[36]. Misfolded chains are usually removed in the endoplasmic reticulum, but in certain conditions, such as viral infection, they accumulate thus generating a cascade of intracellular events including the activation of the protein BiP, the endoplasmic reticulum-unfolded-protein-response (UPR) and the activation of nuclear factor κB (NFκB) which plays a critical role in the induction of inflammation[37]. In contrast with this theory, it has been recently demonstrated that in HLA-B27 over-expressing rats, which normally develop colitis and SpA, the increased expression of β2m prevented colitis but not SpA[38]. In this study over-expression of β2m caused a reduction of HLA-B27 misfolding and an unfolded protein response, suggesting that HLA-B27 heavy chain misfolding may be critical in the development of colitis but not of SpA. In addition to misfolding, data suggest that HLA-B27 heavy chains preferentially form homodimers which bind immunoglobulin-like receptors expressed on the cell surface of natural killer (NK) cells, T cells and monocytes[39]. However, the actual role played by the interaction between HLA-B27 homodimer and its paired receptor in the inflammatory process is still poorly understood. Finally, data suggest that deposition of β2m, caused by the high dissociation rate between HLA-B27 heavy chain and β2m, occurring within synovial tissue, may lead to the initiation of chronic inflammation[40].

COMMON INFLAMMATORY MECHANISMS: FROM THE GUT TO THE JOINTS

The concept that immune cells, activated in the

inflamed gut and migrating into the joints, may be able to reproduce in this tissue a similar immune response, is sustained by the observation that common immunological processes operate at both these sites. Attention has been recently focused on the role of T helper 17 cells (Th17) in IBDs and IBD-related SpA. Th17 cells form a novel class of T helper cells characterized by the expression of the proinflammatory cytokines interleukin (IL)-17A, from which comes the name Th17, IL-17F, IL-22 and TNFα. IL-6 and TGFβ have been shown to be crucial for the differentiation of these cells while IL-23, another proinflammatory cytokine, is thought to be important for their maintenance and expansion[41,42]. Several lines of evidence indicate that Th17 cells may play a role in the induction and maintenance of gut inflammation in CD while their role in UC is still uncertain. Indeed, IL-17A and IL-17F are highly expressed in the gut of patients affected by CD, and Th17 cells have been shown to induce intestinal inflammation in different mouse models of colitis[43-46]. Analogously, high expression of IL-17 was found in the synovial fluids of SpA-affected patients and an increased number of circulating Th17 memory-like T cells has been recently reported in these patients[47,48]. An association between Th17 cells and IBD is further supported by the observation that mutations of IL-23 receptor reduce the risk of developing IBD[49] and the same mutations were found to protect against SpA[50]. Although the functional role of IL-23R mutations remains unclear, the fact that IL-23 signaling plays a critical role in the Th17-mediated inflammation, implicates that Th17 cells may represent a common pathogenetic mechanism in both IBD and SpA.

Tumor necrosis factor-α (TNFα) is a proinflammatory cytokine largely expressed in the lamina propria of patients affected by IBD (mainly CD and to a lesser extent UC), rheumatoid arthritis (RA) and SpA. The role of TNFα in IBD-related SpA has been investigated in the $Tnf^{\Delta ARE}$ mouse model which is characterized by the high expression of TNFα. These mice develop a phenotype dominated by IBD-like intestinal inflammation and arthritis thus implicating TNFα as a required factor for the induction of inflammation in both IBD and IBD-related SpA[51]. In this model, intact TNFα signaling in radiation-resistant mesenchymal cells was found to be required for the induction of SpA as shown by the absence of SpA observed in lethally irradiated TNFαRI knockout mice reconstituted with $Tnf^{\Delta ARE}$ bone marrow cells[52]. Moreover, selective expression of TNFαRI in intestinal myofibroblasts (IMF) and synovial fibroblasts (SF) was sufficient to re-establish intestinal inflammation and SpA in $Tnf^{\Delta ARE}$-TNFαRI knockout mice. MIF and SF expressed high levels of extracellular matrix-degrading metalloproteinase (MMP)-9 and -3 accompanied by reduced levels of the tissue inhibitor of MMPs-1 (TIMP-1) in response to TNFα stimulation which were in part responsible for the tissue damage observed in both the gut and the joints.

COMMON TREATMENT OPTIONS

These studies provide a rationale for developing new strategies for the therapy of IBD-related SpA. However, only the neutralization of TNFα has so far found clinical application in the therapy of IBD-related SpA. An early report showed that two patients affected by CD-associated AS refractory to conventional therapy, experienced amelioration of the axial symptoms after anti-TNFα therapy with infliximab 5 mg/kg intravenously[53]. The efficacy of infliximab in the therapy of SpA was later confirmed by two randomized controlled trials. In a first randomized, double-blind trial 40 patients affected by SpA were randomly assigned to receive either infliximab 5 mg/kg (weeks 0, 2, and 6) or placebo. At 12 wk there was a significant improvement of both the BASDAI (Bath Ankylosing Spondylitis Disease Activity Index) and BASFI (Bath Ankylosing Spondylitis Functional Index) in the infliximab group in comparison to controls[54]. Similar results were obtained by Braun *et al*[55] in a randomized, controlled, multicenter trial in which 53% (18 of 34) patients affected by AS treated with infliximab 5 mg/kg (weeks 0, 2, 6) *vs* 9% (3 of 35) treated with placebo showed a reduction of at least 50% of the BASDAI, BASFI and BASMI (Bath Ankylosing Spondylitis Metrology Index) compared to baseline. However the presence of concomitant IBD in the patients participating in both these trials was unknown. The only trial evaluating the efficacy of anti-TNFα in a cohort of patients affected by CD-related SpA is an open-label study comparing infliximab *vs* conventional therapy[56]. In this study 21 patients with active SpA were enrolled. Sixteen patients with active CD were treated with infliximab (5 mg/kg) at 0, 2, and 6 wk. If remission was achieved patients were treated with a maintenance dose of 3 mg/kg every 6-8 wk otherwise 5 mg/kg was administered. Eight CD-affected patients were in clinical remission at the beginning of the study. These patients were treated with a dose of 3 mg/kg following the same schedule. Twelve additional patients affected by active CD and SpA underwent conventional therapies. Results from this study showed a significant reduction of the BASDAI and spinal pain in the group treated with infliximab in comparison to patients undergoing conventional therapy. Finally, it has been recently suggested that treatment of SpA with infliximab but not etanercept (another anti-TNFα agent) prevents new onset or flares of IBD[57].

The use of adalimumab, a fully humanized anti-TNFα has achieved similar results. A multicenter, randomized, placebo-controlled, trial aimed at assessing the efficacy and safety of 40 mg adalimumab administered subcutaneously for 12 and 24 wk, found that adalimumab was significantly more effective in inducing ASAS20 (20% response according to the ASsessment in Ankylosing Spondylitis International Working Group criteria) than placebo[58]. Moreover the long term efficacy of adalimumab regimen in the treatment of IBD-related SpA has been recently confirmed in a 2-year follow-up study after the initial treatment[59].

CONCLUSION

SpA is a common extraintestinal manifestation of IBD. However, the immunological mechanisms linking gut and joint inflammation are still poorly characterized. The observation that in most of the cases intestinal inflammation precedes SpA has led to the hypothesis that the inflammatory process initially localized in the gut may be "relocated" to a different site. Indeed most of the data summarized here support this concept, providing evidence that T cells and monocytes/macrophages activated by gut-related antigens may be able to home in to the synovial tissue as a result of the expression of adhesion molecules which partially overlap with those expressed by endothelial cells in the gut. In synovial tissue, activation of inflammatory cells may be sustained by several mechanisms including the presence of bacterial antigens and/or by the altered expression of HLA-B27. Finally Th17 cells and high expression of TNFα may play a crucial role in the inflammation-related tissue damage in both the gut and the joints by inducing the expression of extracellular matrix metalloproteinases. However, it is important to note that in some cases, SpA has been shown to precede IBD thus indicating that the illustrated mechanism may not be always applicable and that other immunological processes may link gut inflammation to inflammatory processes localized in different extra-intestinal sites.

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REVIEW

Diet, ageing and genetic factors in the pathogenesis of diverticular disease

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Abstract

Diverticular disease (DD) is an age-related disorder of the large bowel which may affect half of the population over the age of 65 in the UK. This high prevalence ranks it as one of the most common bowel disorders in western nations. The majority of patients remain asymptomatic but there are associated life-threatening co-morbidities, which, given the large numbers of people with DD, translates into a considerable number of deaths per annum. Despite this public health burden, relatively little seems to be known about either the mechanisms of development or causality. In the 1970s, a model of DD formulated the concept that diverticula occur as a consequence of pressure-induced damage to the colon wall amongst those with a low intake of dietary fiber. In this review, we have examined the evidence regarding the influence of ageing, diet, inflammation and genetics on DD development. We argue that the evidence supporting the barotrauma hypothesis is largely anecdotal. We have also identified several gaps in the knowledge base which need to be filled before we can complete a model for the etiology of diverticular disease.

Key words: Diverticular disease; Dietary factors; Genetics; Colon; Inflammation

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INTRODUCTION

Diverticular disease (DD) is characterized by out-pouching in the wall of the colon; though generally benign, a minority of individuals develop associated morbidities, ranging from excessive flatulence and minor Irritable Bowel Syndrome (IBS)-like symptoms, through to inflammation of these out-pouchings (diverticulitis)[1]. Diverticulitis can lead to potentially life-threatening complications (i.e. abscess formation, colonic perforation, and bowel obstruction) in up to a quarter of sufferers[2] and one estimate puts European DD associated mortality at 23 600 deaths per annum[3].

EPIDEMIOLOGY AND PUBLIC HEALTH IMPACT

Necropsy-based studies implicate ageing as the primary risk factor for DD. Two studies in separate Northern European populations, dating from 1968 and 1979, indicate a prevalence of around 13% up to 54 years of age and rising to 40%-50% in individuals over 75 years old[4,5] (Figure 1A). Age-standardized mortality rates for DD in the UK have not changed considerably since 1979[6], so we can assume that these figures are a reasonable estimate of the current prevalence. As an age-related phenomenon we can expect the burden of DD upon society to rise with the continuing increases in life expectancy throughout the developed world (data from

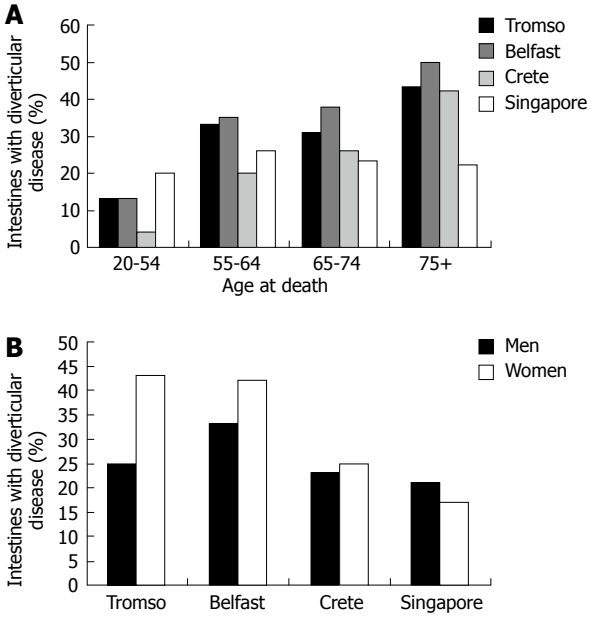

Figure 1 Prevalence of DD in intestines obtained at necropsy by age, gender and region. A: Increasing prevalence of DD with age in western populations. In contrast DD peaked amongst individuals in their late fifties and early sixties in Singapore. The Singapore study however is based on diverse ethnic populations which may be confounding these observations; B: Percentages of intestine shown to have DD present at necropsy by gender. Data obtained on individuals from the Trømso region of northern Norway between 1974 and 1976, Northern Irish subjects in 1968, Cretan subjects 1997-1999, and Singapore prior to 1986. Note: The data presented for the Cretan-, Belfast- and Singapore-based studies are adjusted for age groups used by the Norwegian study using slopes obtained from the published data. Figures adapted from reference[1-4].

the UK Office of National Statistics show continued increases in life expectancy in the UK[7]).

Diverticular disease has been described as a 20th century phenomenon[8], however there are cases in the European literature dating from well before 1900 as evidenced by Jun and Stollman[9]. In addition, evidence of an increase in the death rate from diverticulitis between 1923 and 1966 in England and Wales has been noted[10], and this probably reflects the increasing percentage of elderly people in the population over the same period[7]. The age-standardized mortality rates for DD in the UK have not changed considerably since 1979[3]; it follows that advances in medical practices coupled with increased lifespan may help explain in part the increased diagnosis of DD in the 20th century.

Necropsy data from Northern Europe, Southern Europe and Asia indicate wide geographic disparities in DD prevalence, i.e. it is more common in Northern Europe than in Crete or Singapore (Figure 1A). These studies also show an east-west divide in the nature of disease presentation, with right-sided diverticula being more prevalent in Asian populations; this hints at distinct etiologies[11]. Finally, the necropsy data also indicate that DD is more prevalent in women than men (Figure 1B). It is not yet clear as to whether this gender effect is related to hormonal or anthropometric risk factors, although Manousos *et al*[12] report a relationship with parity.

There have been several studies of DD risk in migrant populations. For example, "non western" immigrants showed a lower risk of DD-related hospital admissions and death but after adjusting for age, risk increased with years of residence in Sweden[13]. In contrast, other studies found no changes in DD incidence amongst migrant communities following periods of naturalization in countries with high or low risk DD in the native population. For example, evidence gathered from endoscopy reports suggests that the predominantly Turkish migrant community in the Zaanstreek region of the Netherlands have a significantly lower incidence of DD than the native Dutch population. Just 7.5% of 387 immigrants examined were shown to have DD, in contrast to 50% of the 5973 "native Dutch". Unfortunately, no information is available on the subjects' diet, how long they had been in the Netherlands or whether the subjects were first, second or third generation immigrants[14]. A UK study observed a lower incidence of DD amongst patients defined as "Indian subcontinent Asian males and females" compared with other ethnic groups. This study showed no difference in DD incidence between first and second generation Asians although numbers were limited[15]. In addition, an autopsy-based study of 1014 cadavers in Singapore showed a significantly higher risk of DD amongst the ethnically Chinese population when compared to the ethnically Malay and Indian populations[16]. The epidemiological data described suggests environmental and genetic components to DD etiology.

PATHOPHYSIOLOGY OF DIVERTICULAR DISEASE

DD presents as pockets within the colon wall, often around points of penetration of the vasa recta through to the luminal side of the muscularis propria[11], possibly because these sites are inherently weak. In western nations diverticula are most common in, though not confined to, the descending and sigmoid colon (left colon). This is in contrast to Asian nations where they occur primarily in the cecum and ascending colon (right colon)[17]. This difference suggests a role for genetic, environmental or lifestyle factors in the etiology of the condition.

At a functional level, the cecum and ascending colon are the primary sites of bacterial fermentation of carbohydrates and proteins which escape small bowel digestion. Microbial action, coupled with anti-peristaltic mixing, maintains a large digestive mass in this segment of the colon; thereby maintaining distention in the longitudinal and circular muscles of this region of the bowel for significant periods. In contrast, the descending colon serves primarily as a holding reservoir for fecal matter prior to excretion. Fecal matter reaching this stage of the colon is significantly reduced in bulk owing to the re-absorption of water and electrolytes, and the depletion of substrate for microbial activity. In addition, movement of bolus through this phase of the colon is subject to increasing voluntary control with variation in intra-luminal pressures throughout the length of the colon (Figure 2).

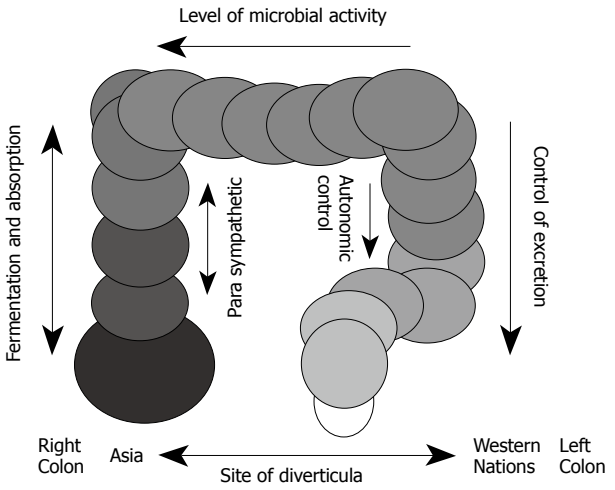

Figure 2 Physiological activity within the large bowel. Schematic of the human colon highlighting functional roles; the right colon is associated with high microbial activity, larger fecal volume and parasympathetic control. DD in the right colon is infrequently observed in western populations but commonly found in Asian populations. The left colon is the primary site of diverticula in western populations and has lower microbial activity, decreased fecal volume and is more responsive to voluntary control.

At a structural level, the mechanical characteristics of the bowel are maintained via circular and longitudinal muscle layers. The circular muscle thickens in regular bands of contraction (plicae circulares) which control peristalsis. The longitudinal muscle also condenses in thick bands (the teniae coli) which serve to pull the colon to a relatively short functional length. In DD, the circular muscle layer is thicker and the longitudinal muscle is shorter[1], although a similar thickening of the colon wall may be a natural feature of the normal ageing bowel[18] and seems to occur at an accelerated rate in inflammatory bowel disease (IBD)[19]. Comparing the DNA to nitrogen ratio in DD tissue confirms that the muscle thickening is not due to hypertrophy[20] whilst individual muscle fiber cells and their organelles appear normal on histological examination[18]. Instead, histological studies suggest that the accumulation and aberrant deposition of connective tissue fibers (elastin[18,21] and collagen[22]) underlie the altered muscle morphology. Furthermore, in diverticulitis the ratio of type I to type Ⅲ collagen is altered in both the serosa and sub-mucosa, indicative of scarring[23]. This effect may be attributable to aberrant activity of matrix metalloproteinases (MMPs) and tissue inhibitors of the matrix metalloproteinases (TIMPs). In one small DD study (11 cases, 6 of which were uncomplicated, *vs* 11 controls) increases in TIMP-1 and -2 expression were associated with disease severity i.e. expression was higher in symptomatic disease[24]. Separately, in a small study of patients with clinical diverticulitis (n = 13), Stumpf *et al*[23] found decreased expression of MMP1. In contrast, Rosemar *et al*[25] found an up-regulation of the expression of MMP1, in addition to increased expression of MMP2 and TIMP1, in DD affected tissues compared to unaffected bowel specimens from the same patients (who were undergoing sigmoid colectomy to treat complicated DD). Whether or not the MMPs and TIMPs play an important role in the tissue organisation observed in asymptomatic DD remains to be seen; the findings reported thus far may be due to acute inflammation rather than DD per se[26].

There have been a number of physiological studies of the diverticular diseased colon, focusing primarily on colonic transit times, intra-luminal pressure, colonic motility and electrophysiology. In the main, inference from these studies about DD specific events or processes is difficult because of the limited data on changes in normal colonic function with ageing, but they offer some insight into the disease process.

Colonic transit times

Studies of colonic transit times (performed by adding radiological markers to the diet) in DD, by both Evans *et al*[27] and Manousos *et al*[28], showed faster colonic transit in individuals with DD. This is perhaps in contrast to what we might expect, given that DD is an age-related phenomenon, and studies of transit time in the aged show either a slower rate of passage through the colon amongst the elderly[29-31], or no differences with ageing[27,32-34]. In addition, Evans *et al*[27] observed longer transit times in females, in whom the evidence points towards a higher risk for developing DD. We might question the potential confounding effects of habitual diet and physical activity in these studies. In particular, upon diagnosis, patients with DD are generally recommended a high fiber diet, which could account for accelerated transit time amongst cases. Nevertheless this interesting counter-intuitive observation is worthy of follow up.

Intra-luminal pressure and colonic motility

Classically, researchers interested in colonic motility in DD performed endoscopy-based manometry studies to measure changes in luminal pressure in the evacuated colon. The main findings of these studies are that there are similar resting luminal pressures between DD cases and controls[35], but higher luminal pressures in segments of colon with diverticula in response to pharmaceutical stimulus[36,37] and an increase in post-prandial colonic motility[38]. Paradoxically, inflating a balloon in the colon of individuals with DD induces the musculature of the colonic wall to yield to the increasing luminal pressure more quickly than in controls[38,39]. In addition, these and later studies indicate increased colonic motility (as assessed by number and amplitude of bowel wall contractions) in the sigmoid colon of individuals with left-sided DD[40,41], and also in the ascending colon of patients with right-sided diverticulosis[42]. These classical studies were generally performed with low numbers and failed to account for age, gender, physical activity or body fat percentage; the physiological observations were made under artificial conditions, i.e. in the evacuated bowel during endoscopy. Furthermore, they were performed over one or two hours, with the subject at rest, whilst in reality one would expect variation in bowel pressures throughout the day. More recently however, Bassotti *et al*[41] made recordings over a 24 h period, and observed higher colonic motility in DD cases throughout the recording period than in a younger control group (cases

Table 1 Summary of electrophysiology studies in diverticular disease (DD)

Study material	Treatment	Response	Study details	Reference
Colonic longitudinal smooth muscle	Relaxative cannabinoid agonists	Decreased relaxation in specimens *vs* controls	Study performed on diverticulitis specimens *vs* colorectal cancer controls	Guagnini *et al*[46]
Colonic longitudinal smooth muscle	Nitroprusside, relaxative agent	Decreased relaxation in specimens *vs* controls	Study performed on 10 DD patients *vs* 10 colorectal cancer controls	Golder *et al*[21]
Colonic circular smooth muscle	Contractionary, cholinergic stimuli	Decreased induction of contractionary waves in cases *vs* earlier data on "normal" patients	Material obtained from 12 patients, 10 of whom exhibited 'abnormal responses' Multiple samples from the same patient showed site specific differences	Huizinga *et al*[103]
Left-sided colonic smooth muscle	Nitric oxide, relaxative agent	Decreased relaxation in cases *vs* controls	Left-sided DD 9 patients, *vs* 16 left-sided colon cancer controls	Tomita *et al*[52]
Colonic longitudinal and circular muscle	Contractory acetylcholine	Increased contractionary response in DD cases *vs* controls	20 subjects with rectal tumours, 10 cases and 10 controls	Golder *et al*[51]
	Contractory tachykinin receptor antagonists	Higher active and resting stress in DD cases		

42-65 mmHg, controls 37-55 mmHg). It is unclear whether or not failing to control for age confounds these studies; Firth and Prather[43] suggest that colonic motility is not altered in the normal ageing colon, but this warrants further investigation, ideally utilizing pressure-sensitive transducers which can be swallowed and allowed to pass through the GI tract to provide more representative measures of colonic physiology[44]. That said, the evidence described points towards a neuromuscular dysfunction in DD; although it remains uncertain whether this is a cause or effect of the condition.

Electrophysiology and neuromuscular dysfunction

Electrophysiological examinations of the bowel wall have been used to investigate neuromuscular dysfunction in DD in several studies. Shafik *et al*[45] identified two distinct types of neuromuscular dysfunction by transcutaneously measuring electrophysiological activity in the sigmoid colons of DD subjects and comparing with age- and sex-matched controls; (1) elevated electrophysiological activity in early stage diverticulosis, and (2) a silent or low electrophysiological tone in advanced DD. This finding is supported by the ex vivo observation of Guagnini *et al*[46] who failed to induce an electrical field twitch response in resected longitudinal muscle from 30% of DD patients, but observed similar responses to electrical field stimulation in the remaining 70% of the samples compared with resected smooth muscle from slightly younger colorectal cancer patients.

Similar *ex vivo* electrophysiological studies of this type on DD specimens are summarized in Table 1. Typically they also show aberrant responses to relaxatory and contractionary stimuli in colonic smooth muscle in DD. On a cautionary note, the weaknesses of these studies include: (1) a lack of power due to the small numbers of subjects; (2) the use of colorectal cancer patients as controls; (3) the focus on complicated/advanced DD specimens.

Evidence from recent histological studies of neurones in the ageing human gut suggests that there is a natural decrease in nerve density with ageing[47,48], a finding supported in animal models[49]. Age-related neurone loss is intuitively attractive as an explanation for the impaired colorectal motility in DD; however, data concerning this remains relatively sparse. An early study by Macbeth and Hawthorne[50] suggested the opposite, i.e. an increase in the number of intramural ganglia but with disorganized distribution of ganglia in DD tissues. Fortunately, recent studies contradict this finding[21], and this work may have been confounded by the morphological distortions associated with the colon shortening in DD. Golder *et al*[51] show histological evidence for decreased nerve content of longitudinal muscle in DD as evidenced by reduced prostaglandin immunoreactivity. They have also found that individual nerve fibers were smaller in cases *vs* controls and were less likely to stain positively for choline acetyltransferase[52] and NOS 1[51], suggestive of cholinergic and nitrergic denervation in these samples. Again the primary potential confounder to these studies is the fact that the colon shortens, and that the muscle layers become thicker (due to elastin and collagen deposition), in DD; it is not clear as to how the authors have controlled for this. In contrast, Bassotti *et al*[53] found no difference in the number of enteric nerves, but a significantly lower number of glial cells in DD. In an interesting take on the same principal, they also found a significant decrease in the number of interstitial cells of Cajal in the myenteric plexus, the sub-mucosa and within the muscle. These cells are emerging as potential colonic pacemaker cells and, like neural cells, their loss might explain poor bowel motility, but yet again we are left with a cause or effect type question. Simpson *et al*[54] have argued (though not evidenced) that nerve damage results from periods of acute inflammation which arise as a consequence of the presence of diverticulosis, whilst others argue that age-related nerve withdrawal induces smooth muscle dysfunction, which thus predisposes to diverticulosis formation[55].

DIET AND LIFESTYLE IN DIVERTICULAR DISEASE

Evidence from man

Seminal papers by Painter and Burkitt[35,36,56] hypothesized

Table 2 Observational studies of dietary fiber consumption and DD risk in man

Design	Findings	Comments	Reference
Case control study comparing dietary fiber intake in 100 (symptomatic) DD cases *vs* 80 age and sex matched controls	Dietary fiber intake significantly higher amongst controls	Study participants were patients hospitalized due to diverticulosis; again symptoms may have influenced their diet	Manousos *et al*[12]
Prevalence of DD assessed by barium enema in 189 non-vegetarian volunteers *vs* 55 vegetarians	Diverticular disease was significantly higher in the non- vegetarian group	"Asymptomatic" volunteers recruited prior to diagnosis and grouped based on dietary choices. A potential confounder is a possible causative effect for meat	Gear *et al*[104]
Case control study comparing dietary fiber intake in 40 (symptomatic) DD cases *vs* 80 age and sex matched controls	Dietary fiber intake significantly higher amongst controls	Dietary fiber intake was "estimated" by dieticians. Symptomatic DD patients were studied so the symptoms may have influenced their diet	Brodribb *et al*[62]
"Prospective" case control study. As part of the Health professionals follow up study, 43 881 US men aged 40-75 followed over 6 years, for self reported diagnoses	Dietary fiber intake significantly lower in cases RR = 0.63	The largest and potentially most informative study. Crucially, the participants were not clinically examined for DD prior to the study. So we cannot rule out effects of DD on dietary choices	Aldoori *et al*[63]
Case control study comparing dietary fiber intake between 86 right-sided DD cases and 106 controls	No relationship between DD and fiber consumption	Only study on right-sided DD, negative finding may indicate either a different etiology or perhaps right-sided DD just has fewer effects that might influence diet choice	Lin *et al*[64]

RR: Relative risk.

that DD arises due to excessive luminal pressures which occur as a consequence of dietary fiber deficiency. This concept was based upon; (1) the apparent increase in DD incidence in western countries throughout the twentieth century, (2) an apparent decrease in dietary crude fiber consumption in western countries over the same period, and (3) an observed low prevalence of DD in Africa where crude fiber intakes were assumed to be higher. In particular, the authors made reference to necropsy studies in Africa which did not record any cases of DD, and to Burkitt's failure to observe any cases of DD whilst working as a clinician in Africa[56]. Subsequently, Painter and Burkitt[8] noted a comparable prevalence of DD amongst African Americans with the white American population.

There are several weaknesses in the evidence base underlying this hypothesis, particularly when one considers the populations being described; we have already evidenced the increasing lifespan of western populations throughout the 20th century which may parallel the increasing prevalence of DD. Life expectancy remains low on the African continent and the most recent World Health Organisation figures report a life expectancy of 51 years for both South Africa and Kenya (the African countries from which necropsy data were referenced by Painter and Burkitt)[57]. Thus there is a smaller percentage of people reaching old age in these countries which would lead to a lower prevalence of DD. Separately, let us consider the dietary fiber intake issue: the necropsy studies cited were performed in the 1950s and so the dietary patterns of the individuals studied would date back to the early half of the twentieth century, making it difficult to accurately determine dietary fiber intakes for these populations. The diet of present day sub-Saharan Africans differs regionally, with urbanization and social standing. For example, the Kenyan staple is the cereal-based "Ugali", fish is common around the coasts, and there are regional preferences for mutton or goat[58]. The average Kenyan diet whilst being relatively high in fiber is also considered, for much of the population, to be total

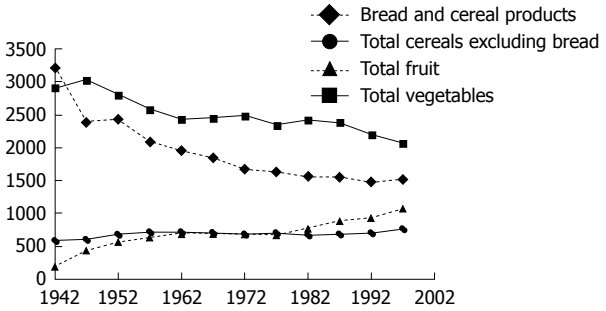

Figure 3 Consumption of major sources of dietary fiber in the UK in grams per person per week since 1942. Data shows a steadily decreasing consumption of dietary fiber-rich foods in the UK population since 1942. Adapted from data produced in the National Food Survey[64].

energy, macro and micronutrient deficient[59]. Similarly, the diet of poor urban South Africans may also be deficient in total energy and some macro and micronutrients, but in contrast to the Kenyan diet it is low in dietary fiber with an increasing emphasis on fat[60]. The point is that modern (and by inference non-traditional) African diets are highly variable; there is also evidence of a shift towards a western dietary pattern with corresponding clinical observations suggesting that DD prevalence may actually be on the rise[61]. However, this observation needs further detailed study and if confirmed, other non-dietary fiber variables should be considered. In the UK, the National Statistics Food Survey (NSFS) dates back to 1942; it shows a steady decrease in the consumption of fiber-rich foods throughout the latter half of the 20th century (Figure 3). However, there has been no great increase in age-adjusted mortality figures from DD in the UK over the last 30 years[3].

A small number of observational studies in man have since attempted to evaluate the dietary fiber/DD risk issue. These studies are summarized in Table 2 and in general support the dietary fiber hypothesis. Two of the three case-control studies[12,62] and the only prospective case-control study[63], found lower dietary fiber consumption amongst cases *vs* controls. In contrast,

one case-control study in an Asian cohort found no link between dietary fiber intake and DD risk (right-sided)[64]. We would argue that all these studies are methodologically flawed; low dietary fiber consumption amongst cases may simply reflect the patients attempt to ameliorate the effects of DD, i.e. excessive flatulence and disordered, possibly aberrant, colonic motility. This is also true of the prospective study[65] in which the volunteers were not clinically examined for DD at baseline, so the study team had no way of knowing whether diverticula were present prior to this. Further, from their cohort of 43 881 male volunteers, aged between 45 and 75, they identified 362 new cases of self-reported symptomatic disease. We would expect the true prevalence of undiagnosed DD in a western cohort (US) of this age and size to be several times that number (Figure 1). A further confounding factor to these studies may be co-linearity (and also inverse linearity) in intake of other nutrients and dietary constituents with fiber consumption. For example, dietary fiber consumption may be inversely related to total energy consumption and hence adiposity[66] and there are independent studies linking an increasing BMI to an elevated risk for complicated DD[65,67]. Unfortunately, there appears to be no published data on obesity and asymptomatic DD. Additional lifestyle risk factors emerging from the literature are: high red meat consumption[12,64], increasing socio-economic status in a Greek cohort[12], hypertension[65], parity[12] and low physical activity with increased symptomatic disease[63]. The evidence base for these risk factors is even less vigorous than it is for dietary fiber. Furthermore, there may be complex relationships between these variables. For instance, low physical activity might be related to obesity and hypertension; it may also be a consequence of symptomatic DD affecting the individual's mobility. Similarly a high socio-economic status in Greece might allow for a higher red meat contribution to the diet, which might itself be inversely correlated to fiber intake. In short, the evidence from studies in man suggests a relationship between diet/lifestyle and DD risk, but there remains a lack of robust definitive evidence. Long-term dietary intervention studies in man aimed at preventing the onset of diverticular disease are unfeasible; there have been intervention trials addressing the benefits of dietary fiber in preventing the complications of DD. These have had mixed success[68,69] and tell us little about disease etiology.

Evidence from animal models

Several investigators have induced the formation of diverticular-like entities in the intestines of rat models through extreme dietary manipulation practiced throughout the animals' natural life-span. Typically these diets were high in animal protein or fat and very low in fiber[70,71]. Increasing the fiber content of these basal diets reduced the DD incidence[72]. Indirectly, these rat models suggest a role for the intestinal flora in DD; Carlson and Hoezel[71] found that they were able to induce DD in rats fed Karaya gum as dietary fiber source, but not in rats where Psyllium seed husks or semi-fibrous cellulose flour was the dietary fiber source. The fundamental difference between these fiber sources may be that Karaya is not well-utilized by the gut microbiota[73]. Furthermore, a maternal high fiber diet throughout gestation in the rat was found to protect the animals' offspring from developing DD in later life[74]. Presumably, this was mediated through maternal colonization of the neonate with a protective microflora, although an epigenetic/imprinting type of effect might also be responsible.

We should, however, consider the validity of the rat model; the rat does not normally develop diverticular diseases and the diets required to induce them were extremely low in fiber. The rat bowel is anatomically distinct from the human; it does not contain teniae coli[74] which have been shown to be abnormal in DD in man. The diverticula observed in the rat model were restricted to the cecum and its proximity, which may be more representative of the Asian phenomenon than the left-sided DD seen in the West. On the other hand, the rat cecal diverticula did show certain similarities to those found in the human in that the muscle wall was thickened with increased deposition of elastic tissue[70] and altered collagen deposition[75]. The rabbit colon does contain teniae and Hodgson[76] induced diverticula in a rabbit model with a long term low residue diet. But again we should consider the validity of the model. The rabbits' natural diet is herbage and they engage in coprophagy to utilize the microbial fermentation products of this rich fiber source. The low fiber diet in this study failed to meet the nutritional needs of the animals and they began to show deficiency type symptoms in addition to developing diverticula. Both models indicate a protective effect for dietary fiber; however in each animal the experimental diets necessarily involve the replacement of dietary fiber with another food constituent; and one could argue that the replacement (fat, carbohydrates and meat protein) components of these diets may be causative of DD in these systems. In line with the studies in man, the animal models suggest that dietary fiber may protect against diverticula development, possibly mediated through the intestinal microflora. Collectively, however, the evidence is poor and the role of other dietary and lifestyle factors remains unexplored.

INFLAMMATION AND DD

It is tempting to postulate that inflammation plays a role in the etiology of DD for several reasons; (1) An increase in plasma inflammatory markers correlates with ageing in man and rats[77,78] as does the prevalence of DD; (2) Bowel wall thickness increases in both IBD[19] and DD[1]; (3) Inflammation could explain neuronal cell death in DD[54]; (4) In a small minority of DD cases the bowel becomes acutely inflamed; (5) Narayan and Floch observed non-specific inflammation in biopsy specimens from non-inflamed DD cases *vs* controls[79]; (6) Kealy observed significantly higher numbers of lymph nodes in disease-free portions of necropsied colon from subjects with DD *vs* controls[80]; (7) Inflammation could be a common factor linking diet and DD.

In contrast, Pezzilli *et al*[81] did not observe differences in fecal calprotectin concentrations between subjects with DD *vs* controls, though the study size was rather small (17 cases). More importantly, recent case-control studies of IBD suggest that chronic acute inflammation actually protects against DD[82,83]. The mechanisms by which this protection occurs are not yet understood, but may involve alterations to the luminal bolus, i.e. the loose watery stool associated with IBD may lower luminal pressures and help prevent DD, or through the impact of intestinal inflammation on the colonic flora. In either case, these findings do not rule out a role for mild non-acute inflammation, with a less pronounced effect on the fecal stream, in DD etiology. However, any mild non-specific inflammation in DD remains poorly evidenced.

THE ROLE OF GENETICS IN DD

Clinical observations associate a number of rare genetic disorders with a strong predisposition towards diverticula formation. Notably, patients with Ehlers-Danlos syndrome[84,85], Williams-Beuren syndrome[86], polycystic kidney disease[87] and Coffin-Lowry syndrome[88] are often afflicted with diverticula of the colon and other internal organs. The etiology of diverticula formation in these syndromes may be unrelated to sporadic age-related DD, but they may offer insight into mechanisms of disease in that at least three of these syndromes are associated with a connective tissue disorder. Ehlers-Danlos syndrome is an inherited connective tissue disorder arising through mutations in either the COL5A1 or COL5A2 genes encoding part of the type V collagen protein or through mutations in the gene for the extra cellular matrix (ECM) protein, tenascin-X[89]. Williams-Beuren syndrome affects 1:10 000 of the population and is due to a deletion of about 20 genes on chromosome 7. Although the genetic basis of this syndrome has not been elucidated fully, it appears to result in elastin haplo-insufficiency[90]. Coffin-Lowry syndrome is a maternally inheritable disorder that may also be related to disrupted collagen metabolism[88]. Scheff *et al*[91] observed colonic diverticulae in 83% of patients with end stage polycystic kidney disease (PKD). PKD is due to mutations in the PKD1 or PKD2 genes coding for the cell membrane-bound polycystin proteins. Whilst the function of these proteins is uncertain, it has been suggested that they interact with the ECM and with extra-cellular signaling pathways regulating cell migration and differentiation[92]. Collectively, these syndromes linked by an ECM defect might suggest that the accumulation of collagen and elastin in the smooth muscle of sporadic DD specimens[20,21] is a prerequisite to diverticula formation.

Separately, clinical observations of poor colonic motility also feature in a significant subset of individuals with mitochondrial diseases[93]. Perez-Atayde *et al*[94] observed a duodenal diverticulum in a 14-year-old with mitochondrial neurogastrointestinal encephalomyopathy which suggests that mitochondrial neuromuscular dysfunction may be associated with DD.

On a different note, clinical case reports hint at familial risk factors for DD in the general population; Schlotthauer reported DD in seven American brothers (aged 40-70), but not in their two sisters (ages not given)[95]; Omojola and Mangete[96] observed DD in three siblings in a Nigerian population with a traditionally low incidence of DD and Claassen *et al*[97] observed DD in two teenage siblings in Holland, in whom they also noted joint hypermobility, perhaps indicating a collagen disorder. Siblings share similar environmental exposure which may help explain familial clustering of DD but does not account for observations in populations where the prevalence is low or in the very young. These observations may simply be statistical anomalies, but taken together with the ethnic variations in both the site and age of onset of DD (Figure 1) they do suggest a genetic component. Unfortunately there is no published literature on attempts to quantify the hereditary component of this condition. Of note, our own preliminary epigenetic data have shown unusual DNA methylation patterns in the colonic mucosa of patients with DD[98,99].

CONCLUSION

A number of questions still remain regarding the biology and etiology of DD. Perhaps the most pressing amongst these relate to the role of diet and lifestyle, as these factors offer strategies for prevention. The two approaches which have been most successful in illuminating the role of diet and lifestyle in DD prevalence thus far are epidemiology and observational studies in man. The epidemiological approach is currently confounded by the lack of available up-to-date data on DD prevalence in different populations. A priority for future research should therefore be the collection of recent necropsy data to indicate current regional prevalence.

There have been no true prospective case-control cohort studies into DD and diet performed to date. For validity, any such study would require a prospective colonic examination to exclude DD patients at baseline, a follow up period of some years and a subsequent exam; this may be unfeasible given the time taken for diverticula to develop and demands on research budgets. In considering a low budget approach, it could be possible to append this type of study to the back of any new prospective colorectal cancer cohorts or to future polyp recurrence trials.

Other clinical and biological questions concern the mechanisms underlying the disease process. Gaps in the knowledge base on the natural changes in bowel physiology, inflammation and composition with ageing impede our understanding of DD. Less invasive methods for measuring physiological activity in the GI tract are under development[44], which may allow for the measurement of colonic motility under more natural physiological conditions and could be employed to study the effects of diet and ageing on normal bowel function and specifically changes related to DD.

Diverticulosis is ultimately a disease of ageing; recent studies show increasing mitochondrial dysfunction in the

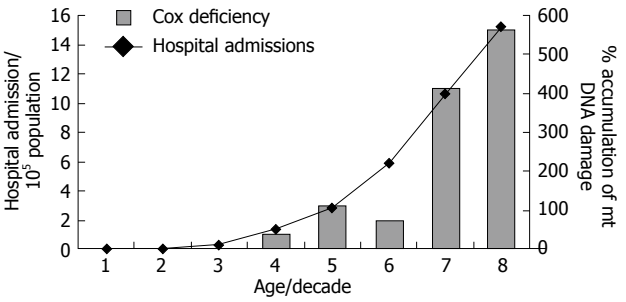

Figure 4 Graph showing the relationship of age with mitochondrial DNA damage and risk of hospital admissions for DD. Graph showing the correlation between the percentage accumulation mitochondrial (mt) DNA damage in the colonic mucosa - right hand y axis whilst left hand y axis shows hospital admissions for DD/10^5 in the UK. Ageing is expressed on the x axis in decades. The incidence of asymptomatic disease is considerably higher but the rate of hospital admissions may mirror DD incidence in the population as a whole. Mitochondrial DNA mutations are inferred from level of deficiency/inactivity in the respiratory chain mitochondrial protein Cytochrome C oxidase[102].

ageing colonic epithelia and this data correlates well with DD prevalence (Figure 4)[100]. We have been conducting preliminary investigations into the accumulation of mitochondrial deficiencies in the colonic epithelia in DD[101], Studies of mitochondrial deficiency or other age-associated changes in the colonic muscle might further illuminate the pathology of this condition. Similarly, we should further examine what phenomenon (if apparently not inflammation) drives excess ECM deposition in ageing and DD.

Animal models of DD hint at a role for the colonic microflora in the disease process, especially when one considers the differences in microflora composition between high and low risk populations[60,102]. A direct comparison of the fecal and colonic mucosal flora between cases and controls might simply reveal differences associated with the altered luminal environment; a better approach would be to characterize the microflora of volunteers prior to any prospective study.

Finally, it remains to be established as to whether right- and left-sided DD have different etiologies and to what extent genetics, environmental and lifestyle factors contribute to this difference. Ageing is the primary risk factor in DD, but the condition is not an inevitable consequence of the ageing process. It seems probable that dietary or environmental factors protect against diverticula formation but further evidence is needed to fully define these factors. The high prevalence of DD within our increasingly elderly population translates into significant morbidity. We feel therefore that the investment of research funds in this area is justified.

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REVIEW

Current prophylactic strategies against hepatitis B virus recurrence after liver transplantation

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Abstract

Prophylactic strategies against hepatitis B virus (HBV) recurrence after liver transplantation (LT) are essential for patients with HBV-related disease. Before LT, lamivudine (LAM) was proposed to be down-graded from first- to second-line therapy. In contrast, adefovir dipivoxil (ADV) has been approved not only as first-line therapy but also as rescue therapy for patients with LAM resistance. Furthermore, combination of ADV and LAM may result in lower risk of ADV resistance than ADV monotherapy. Other new drugs such as entecavir, telbivudine and tenofovir, are probably candidates for the treatment of hepatitis-B-surface-antigen-positive patients awaiting LT. After LT, low-dose intramuscular hepatitis B immunoglobulin (HBIG), in combination with LAM, has been regarded as the most cost-effective regimen for the prevention of post-transplant HBV recurrence in recipients without pretransplant LAM resistance and rapidly accepted in many transplant centers. With the introduction of new antiviral drugs, new hepatitis B vaccine and its new adjuvants, post-transplant HBIG-free therapeutic regimens with new oral antiviral drug combinations or active HBV vaccination combined with adjuvants will be promising, particularly in those patients with low risk of HBV recurrence.

Key words: Hepatitis B virus; Liver transplantation; Recurrence; Prophylaxis; Hepatitis B immunoglobulin

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INTRODUCTION

End-stage liver disease secondary to hepatitis B virus (HBV) accounts for 5%-10% of liver transplantation (LT) performed in the United States and is the leading indication for LT in Asia[1,2]. Recurrence of HBV infection after LT plays a key role in the post-transplant outcomes of the patient and graft, however, in patients with HBV-related disease, complete eradication of HBV after LT is rarely possible. In the 1980s, HBV-related disease was considered a relative contraindication for LT because of poor survival rate and high recurrence rate of HBV in the absence of prophylactic strategies[3]. Thus, prophylactic strategies against HBV recurrence after LT are essential for these recipients. Since the introduce of new antiviral agents and improved prophylactic options, results after LT are reported to be as good or, in a United Network for Organ Sharing (UNOS) database report, even better than in non-HBV patients[4,5]. In this article, current prophylactic strategies against HBV recurrence after LT and evolving new trends are reviewed.

PRETRANSPLANTATION PROPHYLACTIC STRATEGIES

The goals of pretransplant antiviral therapy include the following: (1) to achieve clinical stabilization, thereby delaying/preventing the need for LT; and (2) to attain low HBV DNA levels prior to transplantation, thereby reducing the risk of recurrent HBV after LT.

Interferon (IFN)

Although a major limitation to the use of IFN before LT has been its poor tolerability, it appears to be reasonably well tolerated and effective if patients do not have decompensated HBV cirrhosis. Hoofnagle *et al*[6] have reported that IFN-α therapy stabilized liver function and achieved a sustained loss of HBV DNA and hepatitis B e antigen (HBeAg) in 6 of 18 compensated cirrhotic patients. A prospective study also confirmed that 53 of 103 patients with IFN-α therapy no longer had detectable HBV DNA or HBeAg after a median follow-up of 50 mo[7]. Tchervenkov *et al*[8] also found that pretransplantation IFN therapy followed by hepatitis B immunoglobulin (HBIG) after transplantation was associated with only an 8% recurrence rate after a median follow-up of 32 mo. In addition, a recent study revealed that adjuvant IFN therapy improved the 5-year survival of patients with HBV-related hepatocelluar carcinoma (HCC)[9]. Some data are currently available on the use of peg-interferon α-2b in cirrhosis patients with HBV, after being approved by the US FDA for the treatment of chronic hepatitis B. Chan *et al*[10] have demonstrated that combination treatment of peg-interferon and lamivudine (LAM) led to a higher sustained loss of HBeAg than LAM monotherapy up to 3 years after therapy. Notably, HBeAg or hepatitis B surface antigen (HBsAg) loss is observed more frequently in patients infected with HBV genotype A than with genotype non-A[11,12]. However, the risks associated with IFN therapy and the emergence of safe and well-tolerated oral antiviral therapies have decreased the utility of IFN therapy in patients undergoing LT.

Famciclovir (FCV)

FCV is a guanosine nucleoside analog with activity against herpes viruses and HBV[13]. Several reports[13,14] have described efficacy of FCV in patients with recurrent HBV after LT. However, the number of reports concerning pretransplant application of FCV is limited and the outcome of this pretransplantation prophylactic strategy is not satisfactory. Singh *et al*[15] have found that only 25% of the patients with detectable HBV DNA became pretransplant HBV-DNA-negative after using FCV. Seehofer *et al*[16] also found, in a retrospective study that included 74 HBV-DNA positive patients, that pretransplant FCV did not seem to significantly reduce post-transplant HBV recurrence. Therefore, FCV is rarely used before LT.

LAM

LAM is the first nucleoside analog, a potent inhibitor of HBV replication by competitive inhibition of the reverse transcriptase and termination of proviral DNA chain extension, to be approved for use in HBV treatment, and has an excellent safety profile in both compensated and decompensated cirrhotic patients. The early results using LAM as pretransplant antiviral therapy to suppress HBV replication and improve liver function were promising. Two studies from Villeneuve *et al*[17] and Yao *et al*[18] have reported that serum HBV DNA of all case with positive HBV DNA became undetectable after 6 mo of LAM therapy. The same results were confirmed by other studies[19-21]. These data indicated that LAM monotherapy can achieve the goal of suppression of viral replication to undetectable HBV DNA levels prior to transplantation, and improvement of liver function.

However, the major factor limiting the use of LAM is the development of mutations in the thyrosine-methionine-aspartate-aspartate (YMDD) motif of the HBV DNA polymerase gene, which confers resistance to LAM. In non-immunosuppressed patients, resistance to LAM emerges at a rate of 15%-20% per year, as a result of selection of LAM-resistant mutations in the YMDD motif of the HBV DNA polymerase[22]. As for immunosuppressed patients, LAM resistance can be detected in 45% patients within the first treatment year[23,24]. The sign of resistance is usually a rebound in the HBV DNA level, without other abnormal biochemical or clinical findings, whereas some virological breakthrough caused by antiviral resistance has been reported to cause hepatitis flares and, in rare instances, hepatic decompensation[25,26]. In addition, a retrospective analysis of 309 HBsAg-positive patients listed for LT at 20 North American transplant centers revealed that LAM did not improve LT-free and overall pretransplant survival[27]. LAM is even proposed to be no longer the drug of choice because the initial enthusiasm has been tempered by the high rate of resistance development[28].

Overall, LAM has provided an important treatment option in these patients on the waiting list, with evidence of viral replication or decompensated liver disease related to HBV, but it has turned out not to be the optimal drug and has been proposed to be down-graded from first- to second-line therapy because of its resistance profile.

Adefovir dipivoxil (ADV)

ADV is an oral prodrug of adefovir, a nucleotide analog of AMP, which inhibits HBV DNA polymerase. Previous studies have demonstrated that ADV has excellent activity against wild-type as well as LAM-resistant HBV strains[29-32]. Recently, Marcellin *et al*[33] used ADV administered at doses of 10 mg daily over 48 wk in 171 patients with HBeAg-positive chronic hepatitis B. At week 48, the median change from baseline in HBV DNA was -3.44 log10 copies/mL. Subsequently, 65 patients given ADV 10 mg in year 1 chose to continue in a long-term safety and efficacy study (5 years). The median serum HBV DNA changes from baseline were -2.15, -3.69, -3.55 and -4.05 log10 copies/mL at study weeks 96, 144, 192 and 240, respectively. The median change values from baseline in serum alanine aminotransferase (ALT) concentrations were -43, -18, -49.5, -41 and -50 IU/L at study weeks 48, 96, 144, 192 and 240, respectively, and 66% had normalized serum ALT concentrations at study week 240. As for the resistance to ADV, in the 65 patients with a median of 235 wk (110-279 wk) of ADV exposure, 13 (20%)

Table 1 ADV monotherapy *vs* ADV/LAM combination therapy in patients with lamivudine-resistant chronic hepatitis B

Ref.	Patients (*n*)		Undetectable HBV DNA		Follow up	*P*	Normalization of ALT		Follow up	*P*	ADV resistance		Follow-up	*P*
	A	AL	A (%)	AL (%)			A (%)	AL (%)			A (%)	AL (%)		
[36]	14	28	79	89	At month 36	0.26	73	91	At month 24	0.69	21	0	At month 36	0.020
[37]	23	36	82	89	At month 24	> 0.50	53	79	At month 24	> 0.50	22	0	At month 24	0.001
[38]	28	28	64	40	At month 24	0.38	80	74	At month 12	0.72	18	7	At month 24	0.940
[39]	34	36	82	97	At month 12	> 0.50	79	96	At month 12	> 0.50	18	3	At month 12	> 0.500

A: ADV monotherapy; AL: ADV/LAM combination therapy.

had developed ADV-associated resistance mutations, rt N236T or rtA181V. The first resistance mutation was observed after 135 wk of ADV. In addition, there were no serious adverse events related to ADV. The safety and efficacy of ADV were also confirmed by other studies[34,35].

Thus, ADV has been approved not only as a first-line therapy but also as a rescue therapy for patients with LAM resistance.

ADV and LAM combination therapy

Many of the anti-HBV drugs were initially developed for human immunodeficiency virus (HIV). Resistance develops easily during HIV monotherapy, therefore, it would make theoretical sense that this would also be seen with HBV. The lessons from the HIV field indicate that combination therapy is the way to go, however, we need studies for this in an HBV setting. The above data clearly indicate that ADV monotherapy is effective and safe in waiting-list chronic hepatitis B patients, with or without LAM-resistant HBV, and has much lower rates of resistance than LAM. How effective is ADV and LAM combination therapy in LAM-resistant chronic hepatitis B patients? The latest results[36-39] of ADV alone or in combination with LAM in LAM-resistant chronic hepatitis B are summarized in Table 1. ADV administered in combination with LAM or as monotherapy appeared to be effective in durable suppression of HBV replication and normalization of liver enzymes, and no significant difference was found between these two groups. This result is in accord with the data from a previous study[40], which showed that serum HBV DNA decreased at a similar rate in patients with compensated liver disease and LAM-resistant HBV infection, randomized to ADV monotherapy or combination of ADV and LAM. In addition, one recent study found that short-term (approximately 2 mo) overlap LAM treatment resulted in no better virological and biological outcomes than non-overlap ADV[41]. However, the data concerning incidence of ADV resistance between two groups are controversial. On the one hand, some studies have suggested that there is no obvious improvement in reduction in the development of ADV resistance with ADV alone compared with ADV in combination with LAM[38,39], but one study was limited by its open-label, non-randomized, uncontrolled, retrospective design, and the other study was limited by its short-term follow-up. On the other hand, several recent studies[36,37] have shown that combination of ADV and LAM results in lower risk of ADV resistance than ADV monotherapy. Notably, one was a prospective, randomized controlled study with a small population. Additionally, the same result has been confirmed by other studies[42].

Overall, some of the studies on combination therapy were too short in terms of follow-up, such that differences between monotherapy and combination therapy are not easily distinguished. Thus, long-term, randomized, blinded, controlled clinical trials are still required to determine whether ADV and LAM combination therapy reduces the emergence of ADV resistance compared with ADV monotherapy.

Entecavir

Entecavir is a very potent anti-HBV selective guanosine analog and was approved by the US FDA in 2005, for the management of adult patients with chronic HBV infection. Two early studies[43,44] have suggested that the rates of histological, virological and biochemical improvement, among patients with nucleoside-naive HBeAg-positive or -negative chronic hepatitis B, are significantly higher with entecavir than with LAM, and there is no evidence of viral resistance to entecavir. In addition, several recent studies[45,46] further reinforced this result and a recent randomized international study even found that entecavir therapy resulted in earlier and superior reduction in HBV DNA compared with ADV, in nucleoside-naive HBeAg-positive patients with chronic hepatitis B[47].

Entecavir resistance is associated with the LAM-resistance substitutions M204V/I and L180M, in combination with an additional substitution at residues T184, S202 or M250 in the reverse-transcriptase region of HBV polymerase[48]. In other words, entecavir is associated with a high genetic barrier to resistance that requires multiple mutations for resistance to emerge. In nucleoside-naïve patients, the probability of developing resistance to entecavir remained consistently low (< 1.2%) even after 96 wk of therapy[49]. In contrast, entecavir administration in patients with LAM resistance gives rise to entecavir-resistant mutants. The rate of entecavir resistance after 4 years of treatment of LAM-resistant patients may reach 35%[50]. This results from a particular mode of selection of entecavir strains that follows a two-step process, with the selection of primary resistance mutations at position M204V/I (which are also resistant to LAM), followed by the addition

of secondary resistance mutations to the same viral genomes[51]. Once these secondary substitutions occur, high-level resistance to entecavir occurs.

Generally speaking, as a result of its potency and unique structural formula, entecavir monotherapy represents an interesting first-line treatment option in patients with nucleoside-naive HBeAg-negative chronic hepatitis B, but for LAM-refractory HBV patients, entecavir monotherapy does not appear to be the optimal choice because of the high rate of resistance. To date, there are no specific data available on the use of entecavir in patients in association with LT. Further studies are needed to determine its efficacy and safety profile in this special environment.

Other new antiviral drugs

Telbivudine, which was licensed by the US FDA in 2006, is an oral nucleoside analog with potent and specific anti-HBV activity. It has been demonstrated to be superior to LAM in suppressing HBV DNA in both HBeAg-positive and -negative patients, with less resistance[52-54]. M204I was the only signature mutation associated with telbivudine resistance, in contrast to LAM resistance, which is associated with either the M204I or the M204V mutation. Notably, telbivudine can be used against ADV-resistant mutants. Tenofovir is a nucleotide analog and a potent inhibitor of HIV type 1 reverse transcriptase and HBV polymerase. It was recently approved for the treatment of chronic HBV infection in the United States. Marcellin *et al*[55] reported two studies that compared the antiviral efficacy of tenofovir with that of ADV in both HBeAg-negative and -positive patients. Two of the most encouraging aspects of these two studies are the efficacy of tenofovir in patients with LAM resistance, and the absence of resistance mutations up to week 48. In the treatment of patients with LAM-resistant HBV, tenofovir is superior to ADV and entecavir, and it has a much lower renal toxicity than ADV[56].

However, because of short-term follow up in these studies, cumulative resistance is likely to increase as therapy is extended. Thus, long-term studies are needed to evaluate the safety and resistance of these new antiviral drugs.

POST-TRANSPLANTATION PROPHYLACTIC STRATEGIES

HBIG monotherapy

HBIG was the first agent to show efficacy in preventing HBV recurrence. In 1987, the Hannover group reported that HBIG, to maintain a serum anti-HBs level > 100 IU/L for a minimum of 6 mo after LT, prevented HBV reinfection in liver-transplant recipients[57]. These results were substantiated by a landmark multicenter study from Samuel *et al*[58] in 1993, in which the 3-year actuarial risk of recurrent HBV infection was 75% ± 6% in patients without immunoprophylaxis, 74% ± 5% in those with short-term immunoprophylaxis (2 mo) and 36% ± 4% in those with long-term HBIG prophylaxis (> 6 mo). In a phase 1 clinical study in 2002[59], promising results using a mixture of two monoclonal antibodies to HBV were obtained. A number of mechanisms, which include binding to circulating virions, blocking an HBV receptor on hepatocytes, and promoting antibody-dependent cell-mediated cytotoxicity with lysis of infected hepatocytes, have been proposed to explain the protective effects of HBIG[60,61].

In general, high doses of HBIG (10 000 IU) in the anhepatic phase are followed by daily dosing during the first week after transplantation, and subsequent treatment varies at different centers. Fixed and variable dosing schedules as well as intravenous (IV) and intramuscular (IM) administration have been used[61,62]. A pharmacokinetic study indicated that maintaining anti-HBs titers at > 500 IU/L during the first week post-transplantation, > 250 IU/L during weeks 2-12, and > 100 IU/L after week 12 minimized the risk of recurrence[63].

Despite the successful prophylaxis against HBV recurrence after LT, there are several drawbacks to the use of HBIG. (1) Its high cost, namely up to $100 000 in the first year and $40 000 to $50 000 each year thereafter[64]. (2) Its limited supply. (3) Its side effects. Although HBIG is well-tolerated, significant side effects have been noted, including headache, flushing and chest pain[62]. (4) Escape mutants. Reinfection of HBV in patients receiving long-term use of HBIG can occur because of the development of escape mutants. Mutations in the pre S/S region of the HBV genome can lead to an alteration in the "a" determinant of HBsAg, the primary region of HBV antibody binding, which results in reduced efficacy of HBIG[65-67].

As a result of the above shortcomings of HBIG and the introduction of nucleoside or nucleotide analogs, HBIG monotherapy has vanished from prophylaxis against HBV recurrence after LT. However, HBIG monotherapy may be advocated in some special circumstances. For instance, a recent retrospective study of 639 HBV-infected adult patients undergoing living donor liver transplantation has demonstrated that high-dose HBIG monotherapy resulted in a 5-year HBV recurrence rate of 7.3%[68]. Both Lee *et al*[69] and Takemura *et al*[70] have used HBIG monotherapy as post-transplant prophylaxis against HBV recurrence for patients who received HBsAg-negative/HB core antibody (HBcAb)-positive allografts, with zero recurrence.

LAM monotherapy

LAM monotherapy was the mainstay of prevention of recurrent HBV after LT in the late 1990s and early 2000. Unfortunately, the initial enthusiasm was tempered by the realization that long-term use of LAM, which is essential for maintaining post-transplant viral suppression, is associated with increasing rates of HBV recurrence as a result of drug resistance. Furthermore, immunosuppression has a great influence on drug resistance; LAM resistance was detected in 15% of immunocompetent patients within the first treatment year

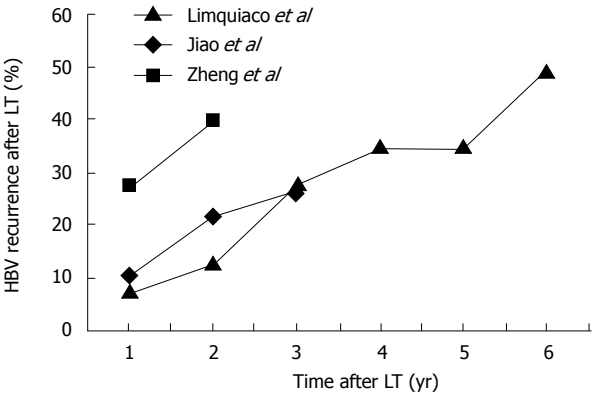

Figure 1 Incidence of recurrent HBV infection after LT using LAM monotherapy as post-transplant prophylaxis. Data adapted from[75-77].

compared with 45% in immunosuppressed patients[23,24]. In early studies, post-transplant HBV recurrence has been reported to be 10% by Grellier *et al*[71], 24% by Lo *et al*[72], 41% by Perrillo *et al*[73], and 50% by Mutimer *et al*[74] at 12, 16, 36 and 36 mo after LT, respectively. Other recent studies with LAM monotherapy have been disappointing with 1-, 2-, 3-, 4-, 5- and 6-year recurrence rates of 8%-27%, 13%-40%, 26%-28%, 35%, 35% and 49%, respectively (Figure 1)[75-77]. As a result of the high rate of LAM resistance and higher risk of recurrence in the graft compared with the combination of LAM and HBIG after LT, this strategy has been abandoned. However, whether combination therapy is required in all patients is unknown. LAM monotherapy has still been advocated by some authors for patients who have received HBsAg-negative/HBcAb-positive allografts, or patients who are HBeAg-negative and have undetectable HBV DNA pretransplantation, because of the low risk of recurrence[73,78,79]. Thus, these patients may be candidates for post-transplant prophylaxis using LAM monotherapy, but further studies with long-term follow-up and a large cohort of patients are necessary to evaluate its efficacy and safety.

High-dose IV HBIG and LAM combination therapy

The use of combination therapy has become a common strategy to overcome the high recurrence rates observed in patients receiving HBIG or LAM alone[80,81]. Mechanisms contributing to the efficacy of this regimen are not well understood, and may be the consequence of the dual effects of reduced production of HBsAg with antiviral therapy, as well as a decreased rate of escape mutations in the pre-S/S and polymerase regions. Combination therapy with high-dose IV HBIG and LAM has been investigated by many centers[82-86], with encouraging outcomes, in that the HBV recurrence rate is < 10% with 1-2 years follow-up. Generally speaking, LAM is commenced pretransplantation, with the aim of reducing the viral load in the peritransplantation period. IV HBIG is given at a dose of 10 000 IU/d for the first postoperative week, and subsequently at a fixed dose of 10 000 IU/mo or with variable dosing to maintain trough anti-HBs titers > 100 IU/L[82-84,87].

Unfortunately, even in patients without overt recurrence of HBV infection, HBV DNA may be detectable by PCR in serum, peripheral blood mononuclear cells or liver tissue in 45% of patients with high-dose IV HBIG 10 years after LT[87]. Similarly, Hussain *et al*[88] recently found that HBV DNA was detected in > 80% of allograft livers in patients who remained serum HBsAg-negative and HBV DNA-negative under combination high-dose IV HBIG/LAM prophylaxis. These data suggest that combination high-dose IV HBIG/LAM prophylaxis cannot eradicate HBV, which also explains the life-long need for HBIG in most patients.

Although combined high-dose IV HBIG and LAM is very effective in preventing recurrent HBV infection, the major limitation of such a regimen is its high cost, estimated at > $100 000 in the first year post-transplantation and > $50 000 yearly thereafter[89]. Other factors including inconvenient administration and unavailability of IV HBIG in some countries limit extensive acceptance of this regimen.

Low-dose IM HBIG and LAM combination therapy

In an attempt to lower the high cost of the combination regimen of high-dose IV HBIG and LAM, new strategies are under consideration. Among these strategies, combination prophylaxis with low-dose IM HBIG has been investigated most extensively, and is regarded as the most cost-effective regimen for the prevention of post-transplant HBV recurrence in recipients without pretransplant LAM resistance. Some studies[77,90-93] concerning this regimen are summarized in Table 2. Recurrence rates reported by major studies[90-93] are similar to those documented with high-dose IV HBIG, and cost reduction by > 50% has led to rapid acceptance of the IM route in many centers. However, a higher rate of recurrence with combined low-dose IM HBIG/LAM prophylaxis was reported by Zheng *et al*[77]. In this retrospective study, 14% developed recurrence at a mean 15.8 mo after LT. The likely explanation is that approximately one-third of patients were high-risk patients, with pretransplantation HBV DNA levels > 10^5 copies/mL. These patients with positive HBV DNA at LT are more likely to develop HBV reinfection after LT. Thus, the number of patients with active viral replication at LT will influence the efficacy of low-dose IM HBIG and LAM combination therapy.

In addition, IM HBIG has been used as long-term maintenance therapy following initial therapy with high doses of IV HBIG[94-96] (Table 3). Although conversion from IV to IM HBIG in combination with LAM can achieve the same prophylactic efficacy as direct low-dose IM HBIG and LAM combination therapy, supplemental IV HBIG is still required in some patients[95,96]. As a result of this inconvenience and the recent finding that HB surface antibody (HBsAb) trough level and half-life do not differ after post-transplantation IV and IM HBIG administration[97], most centers prefer to use low-dose IM HBIG and LAM combination therapy.

Table 2 Prevention of HBV recurrence after LT with LAM and low-dose IM HBIG

Authors	Patients (*n*)	DNA+ prior to LT (%)	Pretransplant LAM therapy (%)	Duration of pretransplant LAM therapy (mean mo)	DNA+ at LT (%)	Prophylactic protocol after LT	Follow-up (mean mo)	Recurrence (%)
Jiao *et al*[90]	79	47	28	0.5	0	LAM + HBIG IM[1]	29	2.5
Gane *et al*[91]	147	85	NA	3	< 50	LAM + HBIG IM[2]	61	4
Zheng *et al*[77]	114	NA	13	5	31	LAM + HBIG IM[3]	16	14
Karademir *et al*[92]	35	51	40	6	14	LAM + HBIG IM[4]	16	5.7
Angus *et al*[93]	32	97	100	3.2	NA	LAM + HBIG IM[5]	18.4	3.1

NA: Not available. [1]2000 IU (IM) at LT, 800 IU (IM) daily for 6 d, weekly for 3 wk, then aim for anti-HBs > 100 IU/L; [2]800 IU (IM) at LT and daily for 6 d, then 800 IU (IM) monthly; [3]2000 IU (IM) at LT, 800 IU (IM) daily for 6 d, weekly for 3 mo, and then monthly; [4]4000 IU (IM) at LT, 2000 IU (IM) daily until anti-HBs > 200 IU/L, then aim for > 100 IU/L; [5]800 IU (IM) at LT and daily for 1 wk, then 800 IU (IM) monthly.

Table 3 Conversion from IV to IM HBIG for prevention of HBV recurrence after LT

Authors	Patients (*n*)	DNA+ prior to LT (%)	Pretransplant LAM therapy (%)	Duration of pretransplant LAM therapy (mean mo)	DNA+ at LT (%)	Prophylactic protocol after LT	Follow-up (mean mo)	Recurrence (%)
Ferretti *et al*[94]	23	48	48	NA	13	LAM + HBIG[1]	20	3.6
Han *et al*[95]	59	NA	59	7.7	8	LAM + HBIG[2]	35	0[3], 2[4]
Faust *et al*[96]	6	NA	0	0	NA	LAM + HBIG[5]	43	0

[1]80 000 IU (IV) in the first wk, then 1200 IU (IM) to aim for anti-HBs > 100 IU/L; [2]IV for a median of 67 wk (LT before August 1998), then IM thereafter; 10 000 IU (IV) at LT, then 10 000 IU (IV) daily for 6 d (LT after August 1998), then IM thereafter; [3]The HBV recurrence of patients with LT before August 1998; [4]The HBV recurrence of patients with LT after August 1998; [5]10 000 IU (IV) at LT, then 2000 IU (IV) for a median of 7 mo, then IM thereafter.

Other post-transplant prophylactic strategies

High costs and inconvenience caused by indefinite HBIG administration have led to controversy as to whether indefinite passive immunization is necessary. In order to stop HBIG after initial monotherapy or combination prophylaxis with LAM, the first approach is to switch from HBIG or HBIG/LAM to LAM monotherapy. The early results were promising. For example, Dodson *et al*[98] switched 16 patients from HBIG to LAM monotherapy after 2 years and had no HBV recurrence 51 mo after LT. In another study by Buti *et al*[99], 29 patients who were HBV-DNA-negative at the time of LT were treated with high-dose HBIG for the first month, and then they were randomized to receive LAM monotherapy (14 patients) or LAM plus HBIG (15 patients) until month 18. None of the patients developed HBV recurrence during the study period. However, with longer follow-up, a recurrence rate of 11%-17% was observed[100,101]. Thus, it is important to determine which patients can stop HBIG. Although it has not yet been defined who can safely discontinue HBIG therapy, the best candidates are probably the following: those without virus replication at the time of transplantation; at least 2 years of HBIG treatment; and negative for HBV DNA by PCR before stopping HBIG.

The second approach is to switch from HBIG/LAM to a combination of antiviral agents. In a recent multicenter randomized prospective study, 16 of 34 patients receiving low-dose IM HBIG/LAM prophylaxis, without HBV recurrence at least 12 mo post-transplantation, were switched to ADV/LAM combination therapy and 18 continued with HBIG/LAM[102]. After a median follow-up of 21.1 mo in the ADV/LAM group and 21.8 mo in the HBIG/LAM group, no patient in either group had HBV recurrence, although one in the ADV/LAM group became HBsAg-positive at 5 mo, but HBV DNA was persistently undetectable by PCR (sensitivity 14 IU/mL). The annual cost of combination ADV/LAM prophylaxis was $8290 *versus* $13 718 for IM HBIG/LAM. Neff *et al*[103] retrospectively investigated a small cohort of non-HBV-replicating patients who were converted from HBIG/LAM to ADV/LAM therapy after a mean post-LT period of 6.5 mo. The mean length of follow-up since therapy conversion was 21 mo. They found that none of the patients showed an increase in transaminases while on dual nucleos(t)ide analog therapy. Unfortunately, there were no results given after the therapy switch, although the authors mentioned that HBV serological testing was performed. Another study[104] has also suggested that this approach may be highly effective and have significant cost savings. In addition, new drugs such as entecavir, telbivudine and tenofovir, are probably candidates to substitute for the indefinite HBIG maintenance therapy after LT. However, available studies are limited, of small size and short follow-up. Thus, larger, randomized prospective studies are required to confirm if combination of antiviral agents is sufficient as a prophylactic strategy against HBV recurrence post-transplantation.

The third approach to prevent HBV recurrence post-transplantation is utilization of active HBV vaccination. Notably, studies regarding this approach have yielded variable results. Successful active immunization in 14 out of 17 hepatitis B patients (82%) after LT was reported by Sanchez-Fueyo *et al*[105] in a cohort of carefully selected low-risk patients. In contrast, in another study, discontinuation of HBIG with a triple course of vaccine produced detectable HBsAb levels in only 18% of recipients[106]. In addition, new hepatitis B vaccines, or

conventional vaccines in combination with new adjuvants are hoped to improve anti-HBs responses in transplant recipients. In a study reported by Bienzle *et al*[107], 16 out of 20 patients (80%) achieved protective antibody titers of > 500 IU/L by using an IM recombinant HBV vaccine combined with two immunostimulants under continuation of passive immunoprophylaxis. However, other studies have failed to replicate this result by using adjuvants and concomitant HBIG administration[108,109]. Therefore, further studies are needed before this approach can be recommended for widespread clinical application.

CONCLUSION

In the setting of pretransplantation, LAM has been proposed to be downgraded from first- to second-line therapy because of its resistance profile. In contrast, ADV has been approved not only as first-line therapy, but also as rescue therapy for patients with LAM resistance. Furthermore, combination of ADV and LAM may result in lower risk of ADV resistance than ADV monotherapy. Other new drugs such as entecavir, telbivudine and tenofovir, are probably candidates for the treatment of HBsAg-positive patients awaiting LT, but long-term studies are needed to evaluate the safety and resistance of these new antiviral drugs.

In the post-transplantation setting, low-dose IM HBIG, in combination with LAM, is regarded as the most cost-effective regimen for the prevention of HBV recurrence in recipients without pretransplant LAM resistance, and is rapidly being accepted in many transplant centers. With the introduction of new antiviral drugs, new hepatitis B vaccine and its new adjuvants, post-transplant HBIG-free therapeutic regimens are promising, particularly in those patients with low risk of HBV recurrence.

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ORIGINAL ARTICLES

Nicotine enhances migration and invasion of human esophageal squamous carcinoma cells which is inhibited by nimesulide

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Abstract

AIM: To study the effect of nicotine on the migration and invasion of human esophageal squamous carcinoma cells and to investigate whether nimesulide can inhibit the effect of nicotine.

METHODS: The esophageal squamous carcinoma cell line (TE-13) was treated with different concentrations of nicotine (100 μg/mL and 200 μg/mL) or 200 μg/mL nicotine plus 100 μmol/L nimesulide. Cell migration and invasion were measured using migration and invasion chamber systems. COX-2 expression was determined by Western blotting. Matrix metalloproteinase-2 (MMP-2) was analyzed by zymography and ELISA.

RESULTS: Nicotine (100 μg/mL, 200 μg/mL) enhanced TE-13 cells migration and invasion, and increased the protein expression of COX-2 and the activity of MMP-2. Nicotine (200 μg/mL) stimulated TE-13 cells migration and invasion which were partly blocked by nimesulide. This was associated with decreased protein expression of COX-2 and decreased activity and protein expression of MMP-2.

CONCLUSION: Nicotine enhances the migration and invasion of the esophageal squamous carcinoma cell line, and nimesulide partly blocks the effect of nicotine-enhanced esophageal squamous carcinoma cell migration and invasion.

Key words: Carcinoma; Cyclooxygenase 2 inhibitors; Esophagus; Nicotine; Squamous cell

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INTRODUCTION

Esophageal carcinoma is relatively common in China, especially esophageal squamous cell carcinoma (ESCC), which has a high mortality rate. It is well established that cigarette smoking increases the risk and mortality of ESCC. Nicotine is a major component of cigarettes. Conventionally, nicotine is regarded as a relatively inert chemical in carcinogenesis. A recent finding suggested that nicotine may at least be partially involved in the initiation, promotion, and even progression of tumors[1,2]. However, the effect of nicotine on tumorigenesis of the esophagus is still not clear.

Many of the critical steps in malignant tumorigenesis, such as cell proliferation, evading apoptosis, stimulating angiogenesis, enhancing cell motility, cell invasiveness and mediating immune suppression, have been associated with cyclooxygenase-2 (COX-2) expression. It has been observed that the expression of COX-2 was increased in ESCC[3] and COX-2 may play a critical role in cancer progression. A number of epidemiological studies have

suggested that the administration of COX inhibitors (NSAIDs, aspirin, indomethacin) reduce the incidence of breast, colon, prostate and esophagus cancers[4-12]. In our previous study, cigarette smoke extract dose-dependently stimulated esophageal squamous carcinoma cell proliferation through up-regulation of COX-2 expression, and a COX-2 inhibitor inhibited this effect. COX-2 inhibitors may decrease the incidence of ESCC, however, the mechanism of COX-2 inhibitors in the metastasis of ESCC is also not very clear.

In order for cancer cells to metastasize, the cells must digest and dissolve the extracellular matrix (ECM) and the basement membrane, which requires the secretion and activation of matrix metalloproteinases (MMPs). The activity of MMPs is associated with invasiveness and metastasis in tumor cells. MMP-2 is a major enzyme that can selectively degrade type Ⅳ collagen and facilitate tumor invasion and metastasis. The expression and activation of MMPs may be directly proportional to the overexpression and activity of COX-2 in tumor cells. A previous study showed that Hs578T breast cancer cells transfected with COX-2 resulted in the activation of MMP-2[13]. Administration of nicotine increased the vascular endothelial growth factor (VEGF)-induced suppression of MMP-2 activity in mice. However, the direct action of nicotine on migration and invasion of esophageal squamous carcinoma cells remains unknown.

In the present study, we evaluated the effect of nicotine on migration and invasion in the human esophageal squamous carcinoma cell line and we investigated whether nimesulide, a selective COX-2 inhibitor, could inhibit migration and invasion in the ESCC cell line treated with nicotine.

MATERIALS AND METHODS

Cell lines and cell culture

TE-13, a human esophageal squamous carcinoma cell line was purchased from Hebei Cancer Hospital of China. Cells were cultured in RPMI-1640 (Hyclone, USA) containing 10% fetal bovine serum (Hyclone, USA). Cells were maintained at 37℃, 95% humidity, and 5% CO_2.

Drug treatment

To examine the effect of nicotine on esophageal squamous carcinoma cells, TE-13 cells were incubated directly with nicotine (Sigma, USA) (100 μg/mL or 200 μg/mL). Dimethyl sulfoxide (0.5%, v/v) was used as a control. TE-13 cells were incubated with nicotine (200 μg/mL) and nimesulide (100 μmol/L) in order to study the effect of the cyclooxygenase-2 (COX-2) inhibitor.

Western blotting for protein

For the detection of COX-2 protein in TE-13 cells, 50 μg protein from the cell extracts of each cell line which had been treated with different drugs for 48 h was electrophoresed through polyacrylamide gel. The separated protein was then transferred to a nitrocellulose membrane (Amersham, USA) and probed with diluted rabbit polyclonal anti-COX-2 (1:200) (Cayman, USA). The next day, after incubation with secondary antibody (Santa Cruz, USA), protein bands on the membranes were then developed by a chemiluminescence detection system (Pierce, USA) and exposed on an X-ray film.

Migration assay

Cell migration assays were performed using a modification of the protocol described by Larkins *et al*[14] and Shin *et al*[15]. The 6.5 mm Transwell® with an 8.0 μm pore polycarbonate membrane insert (Corning Company, USA) was utilized in this assay. The TE-13 cells were harvested and resuspended into serum-free medium containing nicotine (100 μg/mL or 200 μg/mL) or nicotine (200 μg/mL) and nimesulide (100 μmol/L). The upper chamber of the insert was filled with 200 μL of the cells and drug suspension (8×10^4 cells). The lower chamber was filled with culture medium supplemented with 10% FCS as the chemoattractant. The plate was incubated in a humidified environment at 37℃ with 5% CO_2 for 48 h. After incubation, the cells were removed from the upper surface of the membrane by wiping with a moist cotton swab. The migrated cells that passed through the membrane and adhered to the lower surface of the membrane were fixed with methanol, stained for 3 min with hematoxylin and eosin, rinsed with distilled water to remove excess stain not absorbed by cells and counted under a light microscope (× 400).

Invasion assay

Matrigel was purchased from BD Biosciences (USA) and stored at -20℃. After thawing at 4℃ overnight, the matrigel was diluted in serum-free RPMI-1640 medium. 50 μL of the diluted matrigel were evenly inoculated into the upper chamber of the 6.5 mm Transwell® membrane and allowed to form a gel at 37℃. The remaining processes of the matrigel invasive assay were the same as those for the migration assay.

Matrix metalloproteinase-2 activity by Gelatin Zymography

Metalloproteinases are capable of degrading gelatin, therefore, by incorporating gelatin into the polyacrylamide gel a clear zone indicates the presence of a matrix degrading enzyme. Gelatin zymography was carried out as described by Tsujii *et al*[16]. In brief, cells were incubated with nicotine in the absence or presence of nimesulide for 48 h. The supernatants were collected after 48 h and centrifuged 12000 r/m for 10 min at 4℃. The protein content of the supernatant was determined by dye-reagent protein assay. Twenty-one microgram protein from each supernatant was separated on 10% SDS/PAGE with 1 mg/mL gelatin incorporated into the gel mixture. Following electrophoresis at 4℃, the gel was washed with 2.5% Triton X-100 to remove the SDS, rinsed in H_2O three times, and transferred to a bath containing 50 mmol/L Tris-HCl (pH 8.0), 50 mmol/L NaCl and 10 mmol/L $CaCl_2$ at 37℃ for 24 h. To visualize the presence of gelatinolytic bands, gels were stained with Coomassie blue (R-250) and destained with Coomassie

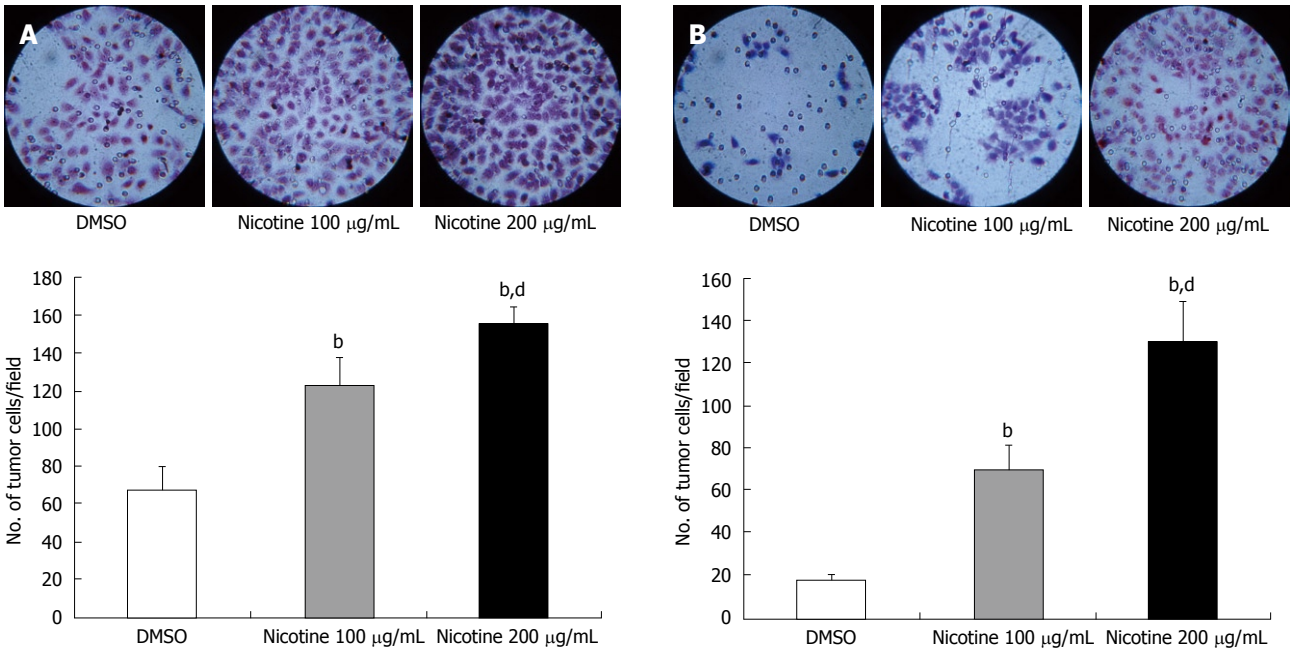

Figure 1 Effect of nicotine on the motility (A) and invasiveness (B) of the esophageal squamous carcinoma cell line TE-13. Numbers of TE-13 cells which migrated through the polycarbonate (A) or matrigel (B) membrane of Transwell® to the lower surface of the membrane were counted under a light microscope (× 400). Columns, mean of three triplicate experiments; bars, standard error of mean. $^{b}P < 0.01$ *vs* the control group; $^{d}P < 0.01$ *vs* the nicotine 100 mg/mL group.

blue destaining solution until all bands of lysis became clear. Quantification of bands on the gels was carried out by video densitometry.

ELISA

We examined the level of MMP-2 in conditioned media from TE-13 cells, using ELISA and commercially available antibodies.

Statistical analysis

Results were expressed as mean ± SE. Statistical analysis was performed using ANOVA. *P*-values less than 0.05 were considered statistically significant.

RESULTS

Effect of nicotine on cellular migration and invasion

Firstly, we tested the effects of nicotine on cellular invasive and migratory potentials. Nicotine (100 μg/mL) induced a 1.8-fold increase and nicotine (200 μg/mL) induced a 2.3-fold increase in TE-13 cellular migration through the 8.0 μm pore polycarbonate membrane of Transwell®, relative to untreated cells ($P < 0.01$) (Figure 1A). Nicotine also increased invasive ability of the tumor cells, and the number of invading cells was increased by 3.6-fold and 6.8-fold in nicotine 100 μg/mL and 200 μg/mL, respectively, compared with the control ($P < 0.01$) (Figure 1B).

COX-2 expression

We examined COX-2 expression in the human esophageal squamous carcinoma cell line (TE-13) following treatment with nicotine or nicotine and nimesulide using Western blotting. Nicotine (100 μg/mL or 200 μg/mL) significantly increased the expression of COX-2 in TE-13 cells when compared with the control group, and the higher concentration of nicotine increased the expression of COX-2 more than the lower concentration of nicotine (Figure 2A). Nimesulide (100 μmol/L) suppressed the increased expression of COX-2 induced by nicotine (200 μg/mL) (Figure 2B).

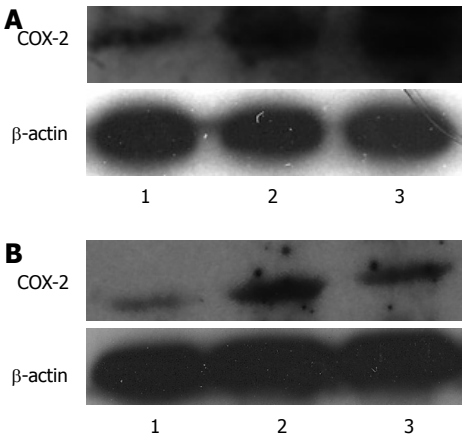

Figure 2 Western blotting analysis. A: Effect of nicotine on COX-2 protein expression in TE-13 cells, 1: DMSO; 2: Nicotine 100 μg/mL; 3: Nicotine 200 μg/mL; B: Effect of nimesulide on COX-2 protein expression in TE-13 cells, 1: DMSO; 2: Nicotine 200 μg/mL; 3: Nicotine 200 μg/mL + Nimesulide 100 μmol/mL.

Effect of COX-2 on tumor cell migration and invasiveness induced by nicotine

To determine if the effect of nicotine on cellular migration and invasion was associated with COX-2 in tumor cells, we examined the effect of the COX-2 inhibitor, nimesulide, on tumor cell migration and invasiveness induced by nicotine. Nimesulide partially,

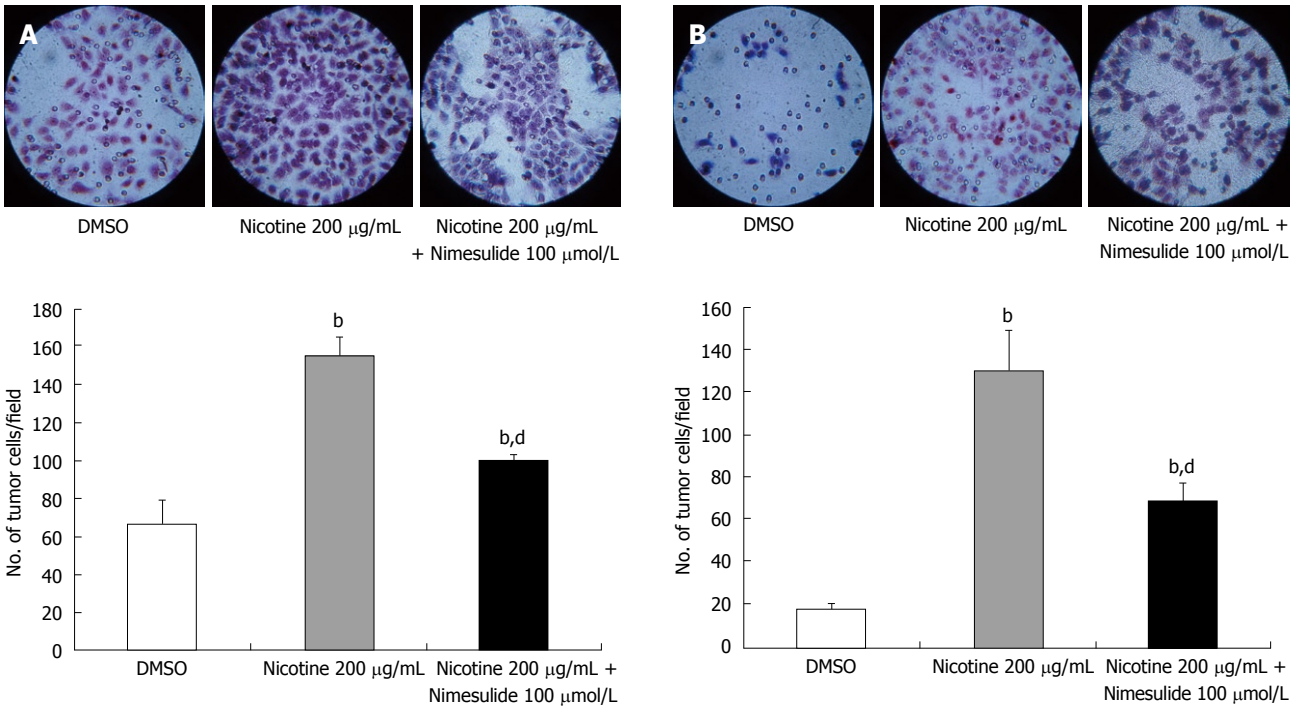

Figure 3 Effect of nimesulide on the motility (A) and invasiveness (B) of the esophageal squamous carcinoma cell line TE-13 induced by nicotine treatment. Numbers of TE-13 cells which migrated through the polycarbonate (A) or matrigel (B) membrane of Transwell® to the lower surface of the membrane were counted under a light microscope (× 400). Data are expressed as mean ± SE. $^{b}P < 0.01$ *vs* the control group; $^{d}P < 0.01$ *vs* the nicotine 200 μg/mL group.

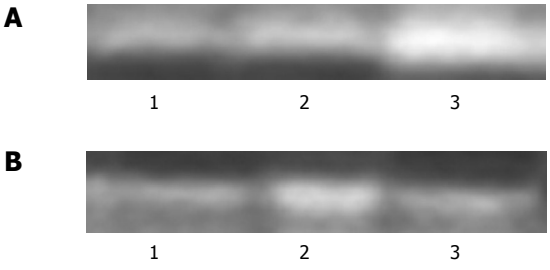

Figure 4 A Gelatin zymography of MMP-2 activity of TE-13. A: Effect of nicotine on MMP-2 activity of TE-13 cells. Our study showed nicotine increased the activity of MMP-2. 1: DMSO; 2: Nicotine 100 μg/mL; 3: Nicotine 200 μg/mL; B: Effect of nimesulide on MMP-2 activity of TE-13 cells treated by nicotine. Our study showed nimesulide suppressed the effect of nicotine that increased the activity of MMP-2. 1: DMSO; 2: Nicotine 200 μg/mL; 3: Nicotine 200 μg/mL + Nimesulide 100 mmol/L.

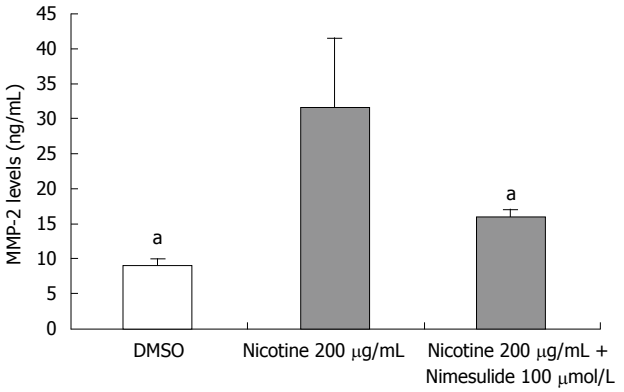

Figure 5 The protein levels of MMP-2 of TE-13 by treatment of nicotine or nicotine and nimesulide were analyzed by ELISA. The experiments were done as described in Materials and Methods. Data are expressed as mean ± SE. $^{a}P < 0.05$ *versus* the nicotine 200 μg/mL group.

but significantly, inhibited the migration of TE-13 cells through the Transwell® membrane (Figure 3A) and significantly inhibited the invasion of TE-13 cells through the matrigel membrane (Figure 3B).

Effect of nicotine on MMP-2 in TE-13 cells

Because activation of MMP-2 can selectively degrade type Ⅳ collagen, which contributes to tumor invasion and metastasis, its activity is an important determinant of tumor cellular invasive potential. We measured MMP-2 activity and expression in TE-13 cells treated with nicotine or nicotine and nimesulide. Nicotine significantly increased MMP-2 (72 kDa) activity (Figure 4A). Nimesulide markedly reduced this increased activity and the protein level of MMP-2 induced by nicotine (Figures 4B and 5).

DISCUSSION

Cigarette smoking causes cancer of various types, including cancers of the lung, oropharynx, larynx, and esophagus. Nicotine, a major component of cigarettes, has been proposed to be responsible for many pharmacological effects of cigarette smoke. Smoking is a neuronal nicotinic acetylcholine (nACh) receptor-mediated addiction[17]. Conventionally, nicotine is regarded as a relatively inert chemical in carcinogenesis and is responsible for the addictive potential of tobacco smoke. However, recently many studies have reported the toxicity of nicotine[18-20] and have suggested that nicotine may at least be partially involved in the

initiation, promotion, and even progression of tumors in the gastrointestinal tract[1,2]. However, reports on the effect of nicotine on esophageal squamous carcinoma are very few. There are three important steps in cancer metastasis: adhesion to the extracellular matrix (ECM), degradation of ECM, and ultimately migration[21]. During the whole process, the motility and invasiveness of tumor cells are the most important characteristics. Therefore, in order to study the effect of nicotine on esophageal squamous carcinoma metastasis, we examined the motility and invasiveness of TE-13 cells, an esophageal squamous carcinoma cell line treated with nicotine. In the present study, nicotine stimulated the motility and invasiveness of TE-13 cells through the reconstituted membrane.

Several studies have reported that COX-2 is involved in these complex steps of cancer metastasis. In Tsujii's study[16], human colon cancer cells (Caco-2) were permanently transfected with a COX-2 expression vector or the identical vector lacking the COX-2 insert. The Caco-2 cells, which constitutively expressed COX-2, acquired increased invasiveness compared with the parental Caco-2 cells or the vector-transfected control cells. Chen *et al*[22] investigated the association between COX-2 expression and colorectal cancer cell invasiveness. Three different colon cancer cell lines, SW620, Lovo, HT-29 and a metastatic variant of HT-29, HT-29/Inv3, were employed to evaluate COX-2 expression and prostaglandin E_2 (PGE_2) production in relation to their invasive abilities *in vitro*. Among the 4 colon cancer cell lines, HT-29/Inv3 manifested the highest COX-2 expression, PGE_2 production and *in vitro* invasive activity. These authors' results implied that COX-2 expression might be associated with the invasive and metastatic properties of colorectal cancer cells. Up-regulation of COX-2 mRNA was observed after exposure to nicotine in human gingival fibroblasts and rat microglial cells[23,24]. Therefore, we studied whether nicotine affected the motility and invasiveness of TE-13 cells through COX-2 up-regulation. Our findings showed that nicotine increased COX-2 expression and nimesulide, a COX-2 inhibitor, partly inhibited the effect of nicotine on motility and invasiveness of TE-13 cells. This implied that the effect of nicotine is at least partly dependent on COX-2 expression. In the study by Shin *et al*[15], nicotine enhanced gastric cancer cell invasion through the matrigel membrane by 4-fold and the effect of nicotine was blocked by a COX-2 inhibitor, which is consistent with our finding.

In order for the cells to invade and migrate through the basement membrane, proteolysis of the extracellular matrix must occur. This is accomplished by the secretion and activation of MMPs, which will degrade all extracellular matrix components. Among MMPs, MMP-2 plays an important role in tumor metastasis. In our study, we also determined the mediation of MMP-2 expression and secretion by nicotine, and used an inhibitor approach to investigate the action of COX-2 on the effect of nicotine. Our findings showed that nicotine increased MMP-2 expression and activity, and nimesulide blocked the effects of nicotine. Several other studies have also implicated that the activity of COX-2 gene expression leads to higher MMP expression. Tsujii *et al*[16] indicated that activation of MMP-2 can be modulated by COX-2 and treatment with a COX inhibitor can reverse the increased invasiveness of Caco-2 cells (which constitutively expressed COX-2) and inhibit activation of MMP-2. Pan *et al*[25] showed that NS398, a COX-2 inhibitor, inhibited MMP-2 mRNA expression, reduced the amount of MMP-2 released into the medium and attenuated the degrading activity of MMP-2. Inhibition of the MMP-2 promoter activity by NS-398 was partially reversed by exogenous PGE2. From these studies and from our findings, we suggest that the effect of nicotine on the stimulation of invasiveness of tumor cells is associated with increased activity and expression of MMP-2 by nicotine, and increased MMP-2 was associated with increased expression of COX-2 by nicotine. Therefore, COX-2 inhibition inhibited the action of nicotine which enhanced the invasiveness of tumor cells by inhibiting the activity of COX-2 and decreasing the activity and expression of MMP-2.

In conclusion, nicotine can enhance the migration and invasion of the esophageal squamous carcinoma cell line (TE-13), and increase the expression of COX-2 and activity of MMP-2 in these cells. Nimesulide partly blocked the effect of nicotine.

COMMENTS

Background

Esophageal squamous cell carcinoma (ESCC) is relatively common in China, and has a high mortality rate. It is well established that cigarette smoking increases the risk and mortality of ESCC. Nicotine is a major component of cigarettes. A recent finding suggests that nicotine may at least be partially involved in the initiation, promotion, and even progression of tumors. However, the effect of nicotine on tumorigenesis in the esophagus is still not clear. It has been observed that an increased expression of cyclooxygenase-2 (COX-2) in ESCC, and COX-2 inhibitors can decrease the incidence of ESCC, however, the mechanism of COX-2 inhibitors in the metastasis of ESCC is not very clear.

Research frontiers

It has been observed that COX-2 may play a critical role in cancer progression. The activity of matrix metalloproteinases (MMPs) is associated with invasiveness and metastasis in tumor cells. The expression and activation of MMPs may be directly proportional to the overexpression and activity of COX-2 in tumor cells. A number of epidemiological studies have suggested that the administration of COX-2 inhibitors can reduce the incidence of breast, colon, prostate and esophagus cancers.

Innovations and breakthroughs

Nicotine can enhance the migration and invasion of esophageal squamous carcinoma cells and nimesulide partly blocked the effect of nicotine.

Applications

COX-2 inhibitors can inhibit the action of nicotine which enhanced the migration and invasiveness of esophageal squamous carcinoma cells. This indicated that COX-2 inhibitors may be an effective preventive and therapeutic strategy for esophagus cancer, however, further investigations are needed to prove this.

Terminology

COX is the rate limiting enzyme involved in the conversion of arachidonic acid to prostaglandin H_2. Two isoforms of COX have been identified which share 60% homology, COX-1 and COX-2. Recently many studies have showed that COX-2 expression is up-regulated in several types of human cancers.

Peer review

In this paper the authors evaluated the effects of nicotine and nicotine and

nimesulide on the migration and invasion in esophageal squamous carcinoma TE-13 cells. The manuscript is interesting and deals with an important subject.

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BRIEF ARTICLES

Antidiabetic therapy and increased risk of hepatocellular carcinoma in chronic liver disease

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Abstract

AIM: To explore the association between hepatocellular carcinoma (HCC) and type 2 diabetes mellitus, describe the temporal relations between the onset of diabetes and the development of HCC and evaluate the possible effects of antidiabetic therapy on HCC risk.

METHODS: We recruited 465 HCC patients, 618 with cirrhosis and 490 control subjects. We evaluated the odds ratio (OR) for HCC by univariate and multivariate analysis. Moreover, OR for HCC in diabetic subjects treated with insulin or sulphanylureas and with metformin were calculated.

RESULTS: The prevalence of diabetes mellitus was 31.2% in HCC, 23.3% in cirrhotic patients and 12.7% in the Control group. By univariate and multivariate analysis, the OR for HCC in diabetic patients were respectively 3.12 (CI 2.2-4.4, $P < 0.001$) and 2.2 (CI 1.2-4.4, $P = 0.01$). In 84.9% of cases, type 2 diabetes mellitus was present before the diagnosis of HCC. Moreover, we report an OR for HCC of 2.99 (CI 1.34-6.65, $P = 0.007$) in diabetic patients treated with insulin or sulphanylureas, and an OR of 0.33 (CI 0.1-0.7, $P = 0.006$) in diabetic patients treated with metformin.

CONCLUSION: Our study confirms that type 2 diabetes mellitus is an independent risk factor for HCC and pre-exists in the majority of HCC patients. Moreover, in male patients with type 2 diabetes mellitus, our data shows a direct association of HCC with insulin and sulphanylureas treatment and an inverse relationship with metformin therapy.

Key words: Hepatocellular carcinoma; Type 2 diabetes mellitus; Insulin; Sulphanylureas; Metformin

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common malignancy worldwide and the third leading cause of cancer-related deaths[1]. In recent years, a significant increase in HCC incidence and mortality rates has been observed in developed countries, but the causes of this growth are only partially understood. Hepatitis C virus (HCV) epidemics certainly play a role, due to the cohort effect of individuals infected in pre-serological age[2]. Although the main risk factors for HCC are HCV, hepatitis B virus (HBV) and chronic alcohol abuse, at least 25% of HCC cases do not have any known etiology, suggesting that further risk factors could be responsible for the increasing incidence of HCC. Diabetes mellitus has recently been proposed as a risk factor for HCC[3]. During the past two decades, the prevalence of diabetes mellitus, and in particular of type 2 diabetes mellitus, has dramatically increased in many countries, including Italy[4]. Sedentary

lifestyles, excessive food consumption and obesity appear to be the main causes of the current diabetes mellitus epidemic in western world[5].

Previous studies on the association between diabetes mellitus and liver diseases showed that type 2 diabetes mellitus appears to be a cause of non-alcoholic fatty liver disease (NAFLD) and that cirrhosis and HCV infection increase the susceptibility to diabetes mellitus[6,7].

Moreover, conflicting results were reported on the association between diabetes mellitus and solid tumors, in particular HCC[3,8-12]. While earlier investigations did not report any association between diabetes mellitus and HCC, recent data clearly indicate that diabetes mellitus is a risk factor of HCC[13-15].

However, the precise relation between diabetes mellitus and chronic liver diseases still needs to be further investigated. Therefore, the aims of this study are to explore the association between HCC and diabetes mellitus in a large cohort of patients with HCC and to describe the temporal relationship between the onset of diabetes and the development of HCC. We also considered the clinical and metabolic characteristics of the patients with type 2 diabetes mellitus and HCC, as well their antidiabetic therapy.

MATERIALS AND METHODS

Ethics

This work has been carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association.

We performed a retrospective, case-control study on three groups of Caucasian individuals, attending the Liver Unit and Diabetic Clinic of 3rd Internal Medicine in the Pordenone General Hospital (Pordenone, Italy) from January 1994 to June 2006. The 3rd Internal Medicine of Pordenone Hospital is a tertiary referral centre for liver disease and diabetes mellitus. This study is a single centre investigation and all patients of the three groups studied were all afferent, directly diagnosed and followed-up in the 3rd Internal Medicine of Pordenone Hospital.

A series of patients with HCC was compared with two different groups: one consisted of patients with liver cirrhosis and the other one, the controls, included individuals who were treated in our Hospital for a wide spectrum of acute conditions.

HCC group

This group comprised 465 consecutive patients with HCC, of which 398 cases (85.6%) were diagnosed by means of cytological or histological examination of hepatic focal lesions. The others (14.4%) were diagnosed according to the following acknowledged criteria[16]: ultrasound examination (also by using micro-bubbles of sulphur hexafluoride as contrast dye in the last three years), α fetoprotein (AFP) > 400 ng/mL, computerized tomography scan and/or magnetic resonance imaging of the upper abdomen. Clinical data, biochemical parameters and the antidiabetic treatment were considered at the time of HCC diagnosis.

Patients with HCC were further divided in two subgroups: the follow-up (FU) and the clinically overt group (CO). The FU group comprised 305 patients with small, single hepatic tumors who received the diagnosis during a surveillance program of HCC in cirrhotic patients based on ultrasound examinations and AFP determinations every 3-6 mo. The clinically overt group (CO) comprised 160 cases with advanced, large size and symptomatic HCC at diagnosis.

LC group

We enrolled 618 patients with liver cirrhosis (LC), matched with HCC cases by age (± 5 years), gender, body mass index (BMI), transaminases, history of diabetes, prevalence of HBV and HCV infections, alcohol consumption and time of admission. These patients were admitted to our Hospital for diagnosis, staging or therapy of liver cirrhosis. Clinical data, biochemical parameters and antidiabetic treatment were considered at the time of recruitment.

According to Child's classification of cirrhosis, patients were classified as follows: class A: 55.5%; B: 24.3% and C: 20.2%. In the cirrhotic patients, the presence of HCC was ruled out through ultrasound examinations, CT or MRI of the upper abdomen and AFP checks.

Control group

From 28740 in-patients of our region, 490 subjects matched with HCC and LC patients by age (± 5 years), gender, BMI, history of diabetes and time of admission were recruited. Those who were admitted for malignancies, alcohol-related disease, viral liver disease and diabetes mellitus were excluded from our study, although comorbidity of these conditions was not considered as an exclusion criterion. As previously reported[15], the selected control group represented our region's general population as to HCV and HBV infections, alcohol consumption and diabetes mellitus prevalence in the age group over 65 years.

Methods

The demographic, clinical and biochemical data of each patient were collected in a computerized database. Biochemical parameters were determined at the Pordenone Hospital central laboratory using standardized and validated methods.

Hepatitis B surface antigen (HBsAg), anti-HBV surface antigen (anti-HBs), anti-HBV core antigen (anti-HBc), and hepatitis B e antigen (HBeAg) were determined using commercial assays (Abbott Diagnostic Division, Wiesbaden; Germany). Sera were also screened for antibodies against HCV (anti-HCV) using a third-generation micro particle enzyme immunoassay (AxSYM HCV version 3.0, Abbott Diagnostic Division). Positive samples were tested for anti-HCV using a third-generation line immunoassay (Immunogenetics, Gent, Belgium) and for serum HCV-RNA using the Roche Amplicor version 2.0 (Roche Molecular System, Pleasanton, CA).

The diagnosis and clinical classification of diabetes mellitus were based on the guidelines of the American Diabetes Association[17,18]. In particular, the distinction

Table 1 Frequency of type 2 diabetes mellitus in HCC, LC and control groups

Subjects (*n*)	DM2 -ve *n* (%)	DM2 +ve *n* (%)	OR (95% CI)	*P*
Total				
HCC(465)	320 (68.8)	145 (31.2)	3.12 (2.2-4.4)[a]	< 0.001
Controls (490)	428 (87.3)	62 (12.7)	2.09 (1.5-2.9)[c]	< 0.001
LC (618)	474 (76.7)	144 (23.3)		
Males				
HCC (364)	246 (67.6)	118 (32.4)	3.14 (2.1-4.6)[b]	< 0.00001
Controls (385)	334 (86.7)	51 (13.3)	1.99 (1.3-2.9)[d]	0.000
LC (450)	341 (75.8)	109 (24.2)		
Females				
HCC (101)	74 (73.3)	27 (26.7)	3.11 (1.3-7.4)[e]	0.002
Controls (105)	94 (89.5)	11 (10.5)	2.59 (1.2-5.9)[f]	0.008
LC (168)	133 (79.2)	35 (20.8)		

OR: Odds ratio; CI: Confidence interval. [a]$P < 0.001$, HCC group *vs* control group; [b]$P < 0.00001$, HCC group *vs* control group; [c]$P < 0.001$, LC group *vs* control group; [d]$P = 0.0002$, LC group *vs* control group; [e]$P = 0.002$, HCC group *vs* control group; [f]$P \leqslant 0.008$, LC group *vs* control group.

between type 1 and 2 diabetes mellitus, was made according to the following clinical characteristics: age and modality of glucose intolerance onset, previous use of antidiabetes medications, occurrence of ketoacidosis, obesity and body fat distribution, concomitant autoimmunity, positive family history and HLA association, presence of micro- and macro-vascular complications when diabetes was diagnosed.

Alcohol abuse was defined as a daily consumption over 30 g for males and over 20 g for females. Average alcohol content was estimated in 5% for beer, 12% for wine and 40% for super alcoholics[15].

Statistical analysis

Normality tests were performed on all data. Parametric data are expressed as mean ± SD. Data with multiple time points variables were analysed by the general model ANOVA. *Post hoc* multiple comparisons were performed using an LSD test when ANOVA testing was significant ($P \leqslant 0.05$). To establish univariate associations among variables, the odds ratio (OR) with a confidence interval of 95% was calculated, using the simple analysis of the logistic regression. All statistical analyses were performed using SPSS software 13.0 for Windows (SPSS Inc, Chicago, IL).

RESULTS

Each subject with diabetes mellitus in the HCC and LC groups showed the clinical and metabolic characteristics of type 2 diabetes mellitus. Of note, none of our HCC or liver cirrhosis patients had type 1 diabetes mellitus.

As shown in Table 1, the prevalence of type 2 diabetes mellitus was 31.2% in the HCC group, 23.3% in the LC group and 12.7% in the controls. The prevalences of type 2 diabetes mellitus in the three groups were statistically different, with an OR of 3.12 (CI 2.2-4.4) in HCC group *vs* controls and an OR of 2.09 (CI 1.5-2.9) in LC *vs* controls group.

Table 2 Etiology, mean age and prevalence of type 2 diabetes mellitus in 465 HCC patients (mean ± SD)

Etiology	HCC *n* (%)	Age (yr)	Prevalence of DM2 (%)
HBV	20 (4.3)	63.3 ± 10.3[a]	3 (15.0)
HCV	177 (38.1)	71.5 ± 7.3[a]	47 (26.5)[b,c]
Alcohol	141 (30.4)	66.7 ± 8.5[a]	52 (36.9)[b,d]
HBV + HCV	8 (1.7)	60.8 ± 12.8[a]	2 (25.0)
HBV + alcohol	9 (1.9)	62.9 ± 9.3[a]	2 (22.2)
HCV + alcohol	81 (17.4)	67.7 ± 9.3[a]	27 (33.3)
HBV + HCV + alcohol	2 (0.4)	68.4 ± 10.3	0
Cryptogenic	27 (5.8)	68.6 ± 9.3	19 (70.3)[c,d]

[a]$P < 0.001$, HCV *vs* HBV + HCV; HCV *vs* HBV; HCV *vs* HBV + alcohol; HCV *vs* HCV + alcohol; HCV *vs* alcohol; [b]$P = 0.048$: HCV *vs* alcohol; [c]$P < 0.001$, cryptogenic *vs* HCV; [d]$P < 0.002$, cryptogenic *vs* alcohol.

Etiology of the chronic liver disease, mean age at HCC diagnosis and type 2 diabetes mellitus prevalence in the subgroups are summarized in Table 2.

The highest prevalence (70.3%) of type 2 diabetes mellitus was found in HCC patients with cryptogenic chronic liver disease.

We also calculated that the male/female ratio in patients with cryptogenic etiology of HCC was 9.5/1, compared to a male excess of 4.4 in all diabetic patients and of 3.3 in the entire HCC group.

Multivariate analysis

The multivariate analysis in HCC group *vs* Controls shows that type 2 diabetes mellitus is associated with an increased risk of HCC occurrence (OR = 2.2; CI 1.2-4.4; $P = 0.01$), independent of age, gender, BMI, alcohol abuse, HBV and HCV infections.

Time interval from type 2 diabetes mellitus onset to HCC diagnosis

The data collected in the records of our Diabetes Clinic show that in 122 patients (84.9%), type 2 diabetes mellitus was diagnosed at least 6 mo before the onset of HCC, while in only 23 patients (15.1%) diabetes mellitus was recognized after the diagnosis of HCC.

The time interval between diabetes mellitus diagnosis and HCC onset was exactly calculated: diabetes was found to be present prior to the HCC diagnosis for a mean time of 141.5 ± 9.4 mo.

Moreover, in patients with HCC of cryptogenic etiology, diabetes mellitus was present before HCC occurrence for a mean period of 150.5 ± 10.1 mo.

In the subgroup with pre-existing type 2 diabetes mellitus, the time interval until the diagnosis of HCC was longer in insulin treated patients than in those treated with antidiabetic oral agents (171.5 ± 87.6 mo *vs* 118.7 ± 95.2 mo; $P = 0.05$).

Insulin treatment

The percentage of type 2 diabetes mellitus patients treated with insulin was similar in the HCC (39.6%) and LC groups (43%), but it was significantly lower in the controls (20.9%; $P < 0.025$ and $P = 0.007$, respectively)

Table 3 Antidiabetic therapy in HCC, cirrhotic patients and in Controls with type 2 diabetes mellitus *n* (%)

	Metformin	Sulfonylureas	Insulin
Total			
HCC	14 (15.9)[a]	74 (84.1)[d]	57 (39.6)[g]
Controls	15 (31.2)[b]	33 (68.8)[e]	14 (20.9)[h]
LC	58 (70.7)[c]	24 (29.3)[f]	62 (43.0)[i]

[a,d] *vs* [b,e] $P = 0.04$; [a,d] *vs* [c,f] $P < 0.001$; [b,e] *vs* [c,f] $P < 0.001$; [g] *vs* [h] $P < 0.025$; [i] *vs* [h] $P = 0.007$.

(Table 3). Based on the records of our Diabetic Clinic, the mean duration of insulin treatment in HCC insulin-treated patients was 83.1 ± 63.4 mo; before insulin therapy the patients were treated only with diet.

Oral antidiabetic agents treatment

We observed that a significant number of HCC patients treated with antidiabetic oral agents took sulphanylureas rather than metformin, while most LC patients used metformin (Table 3). The mean period of treatment with the two antidiabetic oral agents was 125.2 ± 11.5 mo.

Antidiabetic therapy and risk of HCC

Univariate analysis in diabetic HCC patients *vs* diabetic controls shows that the OR for hepatocarcinoma in subjects taking insulin or sulphanylureas was 2.99 (CI 1.34-6.65, $P = 0.007$) while the OR dropped to 0.33 (CI 0.1-0.7, $P = 0.006$) in individuals treated with metformin.

Clinical features of type 2 diabetes mellitus in FU and CO groups with HCC

The FU and CO groups showed a similar prevalence of diabetes (30.2% and 33.1%; $P = 0.62$, respectively). Mean glycated A1c hemoglobin was higher in the FU group (8.2% ± 2.8% *vs* 7.1% ± 2.1%), although this difference is not statistically significant ($P = 0.08$). The mean time interval between type 2 diabetes mellitus diagnosis and HCC onset was greater in CO than in FU patients (167.1 ± 114.3 mo *vs* 127.8 ± 80.1 mo; $P = 0.03$). Insulin treatment was more frequent in FU cases with type 2 diabetes mellitus than in diabetic CO patients (48.9% *vs* 28.3%; $P = 0.01$, respectively).

DISCUSSION

Our study shows that type 2 diabetes mellitus is an independent risk factor for hepatocarcinoma and precedes the onset of HCC. Moreover, we found that insulin or secretagogues antidiabetic oral agents treatment are associated with an increased risk for HCC, while in metformin treated patients the risk of HCC was reduced.

In this study, every HCC and cirrhotic patient with abnormal glucose tolerance showed clinical and pathophysiological characteristics of type 2 diabetes mellitus. This observation confirms the results of two recent prospective studies on the association between diabetes and HCC. El Seragh *et al*[11] in a wide study carried out in the US reported that almost all (99%) diabetic patients who developed HCC had type 2 diabetes mellitus, and Lai *et al*[14] in a survey in Taiwan, observed that type 2 diabetes mellitus, classified according to age at onset of diabetes mellitus, is associated with HCC.

The results of our study are consistent with the theory of the biological mechanisms underlying the epidemiological association between diabetes mellitus and cancer. In fact, this hypothesis postulates that the diabetes-cancer association is likely to be related to insulin resistance and consequent hyperinsulinemia, which are typical features in the majority of type 2 diabetes mellitus patients. Ten years ago, McKeown-Eyssen[19] and Giovannucci[20] observed that risk factors for cancer and insulin resistance in developed countries are almost the same. To explain this analogy, they suggested that protracted exposure to hyperinsulinemia increases the levels of IGF-1, which plays a pivotal role in carcinogenesis (insulin-cancer hypothesis)[21]. In addition, the predictive value of hyperinsulinemia on total cancer mortality[9] and fatal liver tumor incidence[22] has been demonstrated in non-diabetic subjects by two recent prospective analysis.

Liver cirrhosis is a significant cause of death in type 2 diabetes mellitus patients, being even more relevant than cardiovascular diseases[23]. However, patients with type 2 diabetes mellitus often suffer from liver disease as well, and diabetes is a recognized cause of NAFLD and cryptogenic cirrhosis[24]. In fact, it is well-known that the natural history of NAFLD might progress, over a period of many years, from steatosis to steatohepatitis, cirrhosis and, sometimes, to HCC[6]. On the other hand, 20% of patients with cirrhosis have overt diabetes (hepatogenous diabetes) and 60% have impaired glucose tolerance[24]. Thus, the association between diabetes and cirrhosis is complex and reciprocal.

To evaluate the relations between type 2 diabetes mellitus and HCC, regardless of cirrhosis and other risk factors, we performed a single centre, retrospective case-control study on HCC patients comparing them, not only to a group of Control subjects without liver diseases and diabetes mellitus, but also to a series of cirrhotic patients.

Our study shows that the prevalence of type 2 diabetes mellitus in the LC group is intermediate between those of the HCC and controls, indicating that the underlying liver cirrhosis is not the only cause of diabetes in HCC patients.

The similar prevalence of type 2 diabetes mellitus reported in FU and CO subgroups of HCC patients, suggests that the prevalence of diabetes mellitus is not dependent on the size of the liver tumor.

The evidence that type 2 diabetes mellitus is a risk factor for HCC in our patients is obtainable by univariate and multivariate analyses, which show an OR for HCC of 3.12 and 2.2 respectively, similar to those recently reported in U.S.[3,11] and Asian[12,14] populations. Moreover, like the results of a recent Japanese investigation[25], the patients in our study with cryptogenic HCC have the highest percentage of type 2 diabetes mellitus. Interestingly, most patients with cryptogenic HCC are male, suggesting that this sex prevalence could be related to the effect

of diabetes, because the prevalence of type 2 diabetes mellitus, as shown by the current diabetes estimates based on age and sex in the populations of our region[26] and western countries[5,27], is higher in men than in women in the age groups in which HCC develops.

The precise temporal relation between the onset of type 2 diabetes mellitus and diagnosis of HCC is only partially understood. A previous prospective study[11], conducted in a large cohort of males with and without diabetes mellitus, investigated, for the first time, the temporal relationship between diabetes and HCC, showing a two-fold increase of HCC incidence among patients with diabetes.

Our study showed that type 2 diabetes mellitus was present 141 mo before the diagnosis of HCC. Interestingly enough, we observed that in cryptogenic HCC patients, type 2 diabetes mellitus is the only recognized risk factor that these patients have 150 mo before the diagnosis. As already mentioned, patients with small and single hepatocarcinomas and those with advanced tumors have a similar prevalence of type 2 diabetes mellitus. Associated with this, the higher frequency of insulin treatment in FU cases with type 2 diabetes mellitus than in CO diabetic cases, suggests that type 2 diabetes mellitus is more likely to be a cause rather than merely a consequence of the liver cancer.

Our data demonstrates that exogenous insulin treatment is significantly more frequent in diabetic HCC patients than in Control diabetic cases, while cirrhotic patients are more frequently treated with metformin than HCC and Controls individuals. Moreover, in our study, insulin or sulphanylureas therapy are associated with an increased risk of HCC, conversely metformin therapy is associated with a reduced risk of HCC.

These findings are in agreement with previous studies on the relation between antidiabetic therapy and cancer-related mortality, which showed that diabetic patients treated with insulin[9,23] or sulphanylureas[28] have a significantly high mortality for cirrhosis and HCC, while treatment with insulin-sensitizer drugs that ameliorate insulin action, such as metformin, might have a protective effect on cancer risk[29,30].

The effects of insulin therapy in type 2 diabetes mellitus patients with chronic liver disease could be explained by the mitogenic action of exogenous insulin added to those of endogenous hyperinsulinemia. The sulphanylureas might cause an increase both of endogenous insulin secretion and of its precursors, that seem to have mitogenic effects by themselves[21]. Conversely, metformin treatment can decrease insulin resistance, reducing the consequent hyperinsulinemia and its effects in these patients. Moreover, it is known that patients with type 2 diabetes mellitus treated with insulin frequently have more severe insulin resistance, hyperinsulinemia and diabetic complications. Therefore, insulin treatment might be a marker of long lasting and more severe diabetes[31].

The results of our study, therefore, might have important implications in the clinical management of diabetes mellitus, particularly in males with type 2 diabetes mellitus and chronic liver diseases, because we found that they are at high-risk for HCC. This observation is of primary relevance to the implementation of prevention policies and to encourage the most adequate and cost-effective programs of surveillance in cirrhotic patients. In fact, metformin treatment might have a protective effect and therefore might be recommended as a first-line therapy in diabetic patients with compensated liver cirrhosis, because its use is not associated with the risk of hypoglycaemia and body weight gain[24].

Thus, our data suggests that patients with type 2 diabetes and chronic liver disease should first control their diabetes mellitus through diet and changes in lifestyle, to decrease their weight and increase physical activity. Subsequently they should take metformin or other insulin sensitizers, to counteract insulin resistance and consequent hyperinsulinemia.

Our study did have some limitations. It is a retrospective study drawn from a clinical series and not from the community; however, in this case-control survey, matching by age, gender, history of diabetes, BMI and time of hospital admission, we selected a control group and we made sure that it represented the general population of our Region[26]. A potential bias in a case-control study like this is discerning temporal relationships between exposure and outcomes, due to the complex relationships between type 2 diabetes mellitus and cirrhosis. To avoid this error, our study was conducted on a large cohort of HCC patients comparing them with both a control group and with a cohort of cirrhotic patients.

Furthermore, a retrospective study like this must be based upon complete and accurate information on the time interval between type 2 diabetes mellitus onset and the diagnosis of HCC. To achieve this, we reviewed all medical documentation kept at the Diabetes Clinic where all the Regional records are filed from the onset of the disease. Therefore, we could review the detailed clinical history of HCC diabetic patients to calculate exactly the individual time interval from the onset of diabetes to the HCC diagnosis. Prospective studies are required to demonstrate that insulin resistance and hyperinsulinemia are the biological mechanisms that explain the association between type 2 diabetes mellitus and HCC. In conclusion, our survey confirmed that type 2 diabetes mellitus is an independent risk factor for HCC and that it precedes HCC diagnosis. Moreover, in male patients with type 2 diabetes mellitus, our data show a direct association of HCC with insulin and sulphanylureas treatment and an inverse relationship with metformin therapy.

COMMENTS

Background

Type 2 diabetes mellitus has been associated with hepatocellular carcinoma (HCC). However, the relationship between type 2 diabetes mellitus and the underlying liver cirrhosis, and the effects of antidiabetic therapy on HCC risk have not yet been fully evaluated.

Innovations and breakthroughs

This study demonstrates that type 2 diabetes mellitus is an independent risk factor for HCC and pre-exists in the majority of HCC patients. In male HCC

patients with type 2 diabetes mellitus, their data shows a direct association of HCC risk with insulin and sulphanylureas treatment and an inverse relationship with metformin therapy.

Peer review

This is an interesting and detailed retrospective single center study reviewing patients with diabetes mellitus and chronic liver disease.

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BRIEF ARTICLES

Relationship between angiotensin-(1-7) and angiotensin Ⅱ correlates with hemodynamic changes in human liver cirrhosis

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Abstract

AIM: To measure circulating angiotensins at different stages of human cirrhosis and to further evaluate a possible relationship between renin angiotensin system (RAS) components and hemodynamic changes.

METHODS: Patients were allocated into 4 groups: mild-to-moderate liver disease (MLD), advanced liver disease (ALD), patients undergoing liver transplantation, and healthy controls. Blood was collected to determine plasma renin activity (PRA), angiotensin (Ang) Ⅰ, Ang Ⅱ, and Ang-(1-7) levels using radioimmunoassays. During liver transplantation, hemodynamic parameters were determined and blood was simultaneously obtained from the portal vein and radial artery in order to measure RAS components.

RESULTS: PRA and angiotensins were elevated in ALD when compared to MLD and controls ($P < 0.05$). In contrast, Ang Ⅱ was significantly reduced in MLD. Ang-(1-7)/Ang Ⅱ ratios were increased in MLD when compared to controls and ALD. During transplantation, Ang Ⅱ levels were lower and Ang-(1-7)/Ang Ⅱ ratios were higher in the splanchnic circulation than in the peripheral circulation (0.52 ± 0.08 *vs* 0.38 ± 0.04, $P < 0.02$), whereas the peripheral circulating Ang Ⅱ/Ang Ⅰ ratio was elevated in comparison to splanchnic levels (0.18 ± 0.02 *vs* 0.13 ± 0.02, $P < 0.04$). Ang-(1-7)/Ang Ⅱ ratios positively correlated with cardiac output ($r = 0.66$) and negatively correlated with systemic vascular resistance ($r = -0.70$).

CONCLUSION: Our findings suggest that the relationship between Ang-(1-7) and Ang Ⅱ may play a role in the hemodynamic changes of human cirrhosis.

Key words: Renin-angiotensin system; Liver cirrhosis; Angiotensin-(1-7); Angiotensin Ⅱ; Splanchnic circulation; Angiotensin converting enzyme 2

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INTRODUCTION

The renin angiotensin system (RAS) is now viewed as

a dual system composed of 2 arms: vasoconstriction encompassing angiotensin converting enzyme (ACE)-angiotensin (Ang) Ⅱ-Ang Ⅱ type 1 (AT_1) receptor and vasodilation encompassing ACE2-Ang-(1-7)-Mas receptor. The ACE2-Ang-(1-7)-Mas receptor arm mainly acts as a counter-regulatory mechanism to the vasoconstrictor arm[1]. According to this novel concept, the final functional effect of the RAS may reflect a balance between these 2 arms[2-4]. Apart from the circulating RAS, the existence of local systems has been described in a number of organs, including the liver[5]. These local systems act in response to various physiological and pathophysiological stimuli, and the locally generated angiotensins have been implicated in the modulation of cell growth and proliferation, generation of reactive oxygen species, hormone secretion, and in the control of local inflammation and fibrosis[6]. The circulating RAS is well recognized for its role in hemodynamic regulation through Ang Ⅱ, a potent vasoconstrictor, and the counter-regulatory peptide, Ang-(1-7), a vasodilator. The local and circulating RAS can interact with each other and with other regulatory systems[7].

Liver cirrhosis has 2 major circulatory dysfunctions: portal hypertension and a hyperdynamic circulation characterized by elevated cardiac output and low systemic vascular resistance[8]. It is well established that Ang Ⅱ plays a role in the pathogenesis of portal hypertension by increasing intrahepatic vascular resistance and also by contributing to liver fibrosis[5]. The development of portal hypertension in cirrhosis is associated with arterial vasodilation in the splanchnic circulation, leading to a decrease in systemic vascular resistance[9]. Early in the course of the disease, the decrease in systemic vascular resistance is compensated by the development of a hyperdynamic circulation[10]. Despite a reduction in systemic vascular resistance, the effective arterial blood volume remains normal, as does the circulating RAS components and antidiuretic hormone[8]. However, as the disease progresses and arterial vasodilation increases, the hyperdynamic circulation is insufficient to correct the effective arterial hypovolemia[8]. Arterial hypotension develops, leading to activation of the circulating RAS, sympathetic nervous system and secretion of antidiuretic hormone[8]. The splanchnic circulation is resistant to the effect of Ang Ⅱ, noradrenaline and vasopressin, and the maintenance of arterial pressure is a result of vasoconstriction in extra-splanchnic vascular areas[8]. Liver cirrhosis has been studied recently in light of the new view of the RAS. It is becoming clear that the RAS can influence liver cirrhosis through its 2 main arms. While the ACE-Ang Ⅱ-AT1 receptor arm contributes to liver tissue injury and fibrosis[6] and the maintenance of basal vascular tonus in non-compensated cirrhosis[11], the activation of the ACE2-Ang-(1-7)-Mas arm exerts antifibrotic actions[6,12,13]. In addition, it has been speculated that this counter-regulatory arm also has a role in the arterial vasodilation in liver cirrhosis[14]. In this regard, we have recently shown that chronic treatment with propranolol in cirrhotic patients was characterized by marked changes in the precursors of the RAS cascade (renin and Ang Ⅰ), with repercussions on the 2 main RAS components, Ang Ⅱ and Ang-(1-7), in the splanchnic and peripheral circulations[15]. Our previous data suggested that a possible therapeutic approach for advanced human cirrhosis could be the combination of a beta-blocker with an AT_1 receptor blocker or ACE inhibitor[15].

Therefore, the aim of the present study was to evaluate circulating levels of angiotensins in mild-to-moderate and advanced stages of human cirrhosis without the interference of any kind of RAS blockade such as beta-blockers, ACE inhibitors and AT_1 receptor blockers. In addition, we also evaluated a correlation between RAS peptides and hemodynamic parameters in systemic and splanchnic circulations of cirrhotic patients during liver transplantation.

MATERIALS AND METHODS

Patients

This cross-sectional study used a convenience sample recruited from the Alfa Institute of Hepatology/Liver Transplantation and the Clinical Primary Care Center of our institution.

Inclusion criteria: Patients diagnosed with hepatic cirrhosis (n = 24) defined through liver histopathology and/or ultrasonography findings were included in this study. Table 1 displays the Child-Pugh scores[16] of our patients. The primary etiology of the liver disease was established in 21 subjects (87.5%), and included alcoholism, hepatitis C virus, hepatitis B virus and biliary cirrhosis. All cirrhotic patients showed portal hypertension at the time criteria were set for each protocol group. Cirrhotic patients were then allocated to one of 3 study groups based on the presence or absence of ascites and values of Child-Pugh score[16]: the first group was composed of patients who had mild-to-moderate liver disease (MLD, n = 8), the second group included patients with advanced liver disease (ALD, n = 7), which were seen in an outpatient clinic and the third group was composed of liver transplant recipients during surgery (LT, n = 9).

The MLD patients did not receive any medication and did not exhibit ascites at the time of blood collection. However, they had other endoscopic or ultrasonographic signs of portal hypertension (small varices < 5 mm, collateral vessels, abnormalities in portal flow direction).

The ALD group comprised outpatients with ascites and extra-hepatic complications such as encephalopathy and moderate to large esophageal varices (> 5 mm) with risk of bleeding. These patients were using diuretics (furosemide 40-80 mg/d associated with spironolactone 25-100 mg/d).

The LT group included hospitalized cirrhotic patients with the same severity of liver disease as the ALD group based on Child Pugh and MELD scores (Child Pugh: 11.0 ± 0.8 in LD *vs* 11.2 ± 1.2 in LT and MELD: 29.3 ± 2.1 in LD *vs* 29.8 ± 3.2 in LT, $P > 0.05$ for both comparisons). These patients also presented the same clinical and laboratory features as the ALD group and

Table 1 Subject characteristics and casual measurements

Characteristics and measurements	Mild to moderate liver disease (MLD) $n = 8$	Advanced liver disease (ALD) $n = 7$	ALD during Liver transplantation (LT) $n = 9$
Age (yr)	55.5 ± 1.8	54 ± 5	50 ± 3
Sex, male/female	3 (37.5%)/5 (62.5%)	4 (57%)/3 (43%)	7 (78%)/2 (22%)
Child Pugh Score	6.7 ± 0.2	11 ± 0.8[a]	11 ± 1.8[a]
Albumin, g/dL	2.9 ± 0.15	2.4 ± 0.3	2.6 ± 0.2
Bilirubin, mg/dL	2.4 (1.7-5.9)	2.5 (1.2-7.1)	3.3 (2.0-5.5)
Creatinine, mg/dL	0.70 (0.70-0.80)	1.0 (1.0-1.45)[a]	1.0 (0.75-1.50)
INR (International normalization ratio)	1.2 ± 0.07	1.7 ± 0.3	1.8 ± 0.2
Prothrombin activity	68.3% ± 7.5%	50% ± 12%	42% ± 7%[a]
Serum Na^+, mEq/L	139 ± 2	126 ± 3[a]	130 ± 2[a]

Data are expressed as mean ± SE or median (25 and 75 percentile), except for sex where number of patients and percentages are shown. [a]$P < 0.05$ for the comparison of the ALD and LT groups with the MLD group (ANOVA followed by Bonferroni test for mean comparisons and Kruskal-Wallis followed by the Dunn test for median comparisons). No statistical differences were detected between the ALD and LT groups.

received the same diuretic treatment. The only difference between both groups was that LT patients had been submitted for liver transplantation.

The control group ($n = 16$) consisted of healthy age-matched subjects from our Clinical Primary Care Center. Health status was determined through the subjects' medical history to rule out the presence of chronic or acute diseases. All subjects were subjected to a complete physical examination prior to blood sampling as part of our study protocol.

Exclusion criteria: Co-morbidities such as diabetes, heart, pulmonary, autoimmune and neurological diseases automatically excluded subjects from the study. Patients receiving chronic treatment with ACE inhibitors, angiotensin receptor blockers, renin inhibitors, beta-blockers and corticosteroids were also in our exclusion criteria. During liver transplantation, blood collection was performed whenever the subject showed acute hemodynamic derangement demanding vasoconstrictor use.

Ethical aspects: The Ethics Committee of the Federal University of Minas Gerais approved the study. Informed consent was obtained from all included subjects. The research protocol did not interfere with any medical recommendations or prescriptions. Subject follow-up was guaranteed even in cases of refusal to participate in the study.

Study protocol

The study was performed in outpatients at different clinical stages of cirrhosis (MLD and ALD groups) and in hospitalized patients during liver transplantation (LT group). Circulating RAS components levels were measured in all groups; only in LT patients were measurements of RAS components also obtained from the splanchnic circulation simultaneously with the hemodynamic parameters.

Protocol 1-Evaluation of circulating RAS in controls and patients with mild-to-moderate and advanced cirrhosis: Blood samples for measurement of plasma renin activity (PRA) and angiotensins were obtained from healthy subjects, MLD and ALD patients on a single occasion taking into account the inclusion and exclusion criteria for each group. For ethical reasons, no changes to the clinical approach were made for study purposes. Blood samples (10 mL) were collected through peripheral venipuncture in the morning after a fasting period of 8 h. All subjects rested in the supine position for at least 30 minutes before blood sampling.

Protocol 2-Evaluation of peripheral and splanchnic RAS components during the pre-anhepatic stage of liver transplantation: Anesthesia for liver transplantation was induced by a rapid sequence of etomidate, fentanyl and succinylcholine and maintained by isoflurane (CAM about 1.0) and atracurium until blood sampling. In the LT group, blood sampling was performed during the pre-anhepatic stage of liver transplantation and samples were obtained simultaneously from the radial artery (10 mL) and portal vein (10 mL) to evaluate RAS components before and after the enteric circulation, respectively. Furthermore, the portal vein is part of the splanchnic circulation, which is the original source of hyperdynamic circulation.

Protocol 3-Evaluation of hemodynamic parameters during the pre-anhepatic stage of liver transplantation: Hemodynamic parameters (cardiac output, cardiac index, systemic vascular resistance and systemic vascular resistance index) were determined simultaneously with blood sampling to measure the RAS components. These measurements were obtained through invasive continuous monitoring *via* a Swan-Ganz catheter (CCOMBO/SvO_2, 110 cm/7.5F, Edwards Lifesciences, Irvine, CA, USA), using Dixtal (DX 2020, Dixtal Biomedical, São Paulo, Brazil) and Vigilance (CEDV, Edwards Lifesciences, Irvine, CA, USA) monitors.

Blood collection and plasma extraction

For all blood collection, samples were drawn into 2 sets of ice-cooled tubes-one containing 7.5% EDTA for PRA determination and the other containing a cocktail of pro-

Table 2 Circulating RAS components in healthy controls, MLD and ALD patients

RAS components	Healthy controls	MLD	ALD
PRA (ng Ang I/ mL per hour)	0.10 (0.03-0.23)	0.10 (0.01-0.22)	2.7 (0.43-6.61)[a,c]
Ang Ⅰ (pg/mL)	179.8 (86.9-220.8)	28.9 (23-65.2)[a]	412.3 (326.5-1123)[a,c]
Ang Ⅱ (pg/mL)	47.0 (41.8-61.7)	27.5 (23.9-35.9)[a]	84.4 (60.9-154.6)[a,c]
Ang-(1-7) (pg/mL)	20.1 (17.1-25.5)	24.9 (21.1-27.4)	32.6 (27.8-61.6)[a]

Data are expressed as medians (25 and 75 percentile). [a]$P < 0.05$ for the comparison of MLD and ALD groups with healthy controls and [c]$P < 0.05$ for the comparison between MLD and ALD groups (Kruskal-Wallis followed by Dunn test). PRA: Plasma renin activity; Ang: Angiotensin.

tease inhibitors for angiotensin measurements[17]. Blood samples were centrifuged at 2000 *g* for 20 min at 4℃ and plasma was stored at -20℃[17]. Plasma samples were extracted using Bond-Elut cartridges (Analytichem International, Harbor City, CA), as described elsewhere[17].

Radioimmunoassays

PRA as well as Ang Ⅰ, Ang Ⅱ and Ang-(1-7) concentrations were determined through radioimmunoassays, as detailed elsewhere[17]. The recovery of ^{125}I-labeled Ang Ⅰ, Ang Ⅱ, and Ang-(1-7) was 79.2% ± 2.3%, 86.9% ± 0.8% and 83.5% ± 0.9%, respectively. Results were expressed as nanograms of Ang Ⅰ generated per milliliter of plasma per hour (ng Ang Ⅰ/mL per hour) for PRA and pg/mL of plasma for Ang measurements).

Statistical analysis

The software Graphpad PRISM, version 4.03, was used for the statistical analyses. The Gaussian distribution of the variables was evaluated by the Shapiro normality test. Results were reported as mean ± SE or median, when appropriate. Analysis of variance followed by the Bonferroni test was used for the comparison of means between groups. Mann-Whitney or Kruskal-Wallis followed by the Dunn test was used to compare non-parametric data. The paired Student *t*-test was used to compare means from variables of the LT group before and after the enteric circulation. The level of significance was set at $P < 0.05$.

RESULTS

Subject characteristics and casual measurements

The primary etiology of liver disease of the compensated cirrhotic patients (CLD) included: hepatitis C virus in 3, alcoholism in 4 and hepatitis B virus in 1 patient. Laboratory data, Child-Pugh and MELD scores confirmed the mild to moderate stage of liver disease (Table 1).

The causes of liver disease in the ALD group were: alcoholism in 2, bile cirrhosis in 1, hepatitis C virus in 1 and idiopathic disease in 3 patients. Laboratorial data, Child-Pugh and MELD scores revealed the advanced stage of liver disease (Table 1).

The etiologies of hepatic disease in the LT group included hepatitis C virus in 3, alcoholism in 3, bile cirrhosis in 2 and hepatitis B virus in 1 patient. Laboratory findings, Child-Pugh and MELD scores were very similar to those of the ALD group and also showed an advanced stage of liver disease (Table 1).

Healthy controls comprised 16 subjects, including 7 males and 9 females from 40 to 65 (49.7 ± 2.6) years.

Circulating RAS profile in healthy controls, MLD and ALD outpatients

As displayed in Table 2, PRA was significantly higher in the ALD group in comparison to MLD patients and healthy controls ($P < 0.05$). The same profile was observed for Ang Ⅰ, which also presented a significant increase in plasma levels in ALD patients when compared to controls and the MLD group (Table 2). On the other hand, statistical differences were detected when comparing Ang Ⅰ measurements obtained from MLD patients and healthy controls (Table 2).

ALD patients also exhibited a significant elevation in Ang Ⅱ and Ang-(1-7) when compared to healthy controls ($P < 0.05$, Table 2). In contrast, Ang Ⅱ levels were significantly lower in patients with mild-to-moderate cirrhosis even when compared with healthy controls, whereas plasma Ang-(1-7) in this group did not differ from that of healthy subjects or of the ALD group (Table 2).

Ratios of Ang-(1-7) and Ang Ⅰ levels, of Ang Ⅱ and Ang Ⅰ, and of Ang-(1-7) and Ang Ⅱ in all groups are displayed in Figure 1 as medians. The Ang-(1-7)/Ang Ⅰ ratio was significantly higher in MLD patients [0.95 (0.43-1.02)] than in the ALD and control groups. On the other hand, healthy controls and ALD patients exhibited similar Ang-(1-7)/Ang Ⅰ ratios [controls: 0.13 (0.08-0.22) *vs* NLD: 0.08 (0.02-0.10), Figure 1]. The Ang Ⅱ/Ang Ⅰ ratio was significantly reduced in the ALD group [0.15 (0.13-0.25)] when compared to MLD patients [0.98 (0.71-1.09)], but not in comparison to healthy controls [0.25 (0.20-0.63), Figure 1]. More importantly, the Ang-(1-7)/ Ang Ⅱ ratio, which could represent the final functional relationship between RAS components, was significantly increased in MLD patients [0.89 (0.73-1.04)] in comparison to ALD patients [0.40 (0.17-0.57)] and controls [0.38 (0.32-0.47)], whose median values were similar (Figure 1).

Peripheral and splanchnic RAS components during the pre-anhepatic stage of liver transplantation

As displayed in Table 3, the comparisons of PRA, Ang Ⅰ and Ang-(1-7) levels revealed no difference between the peripheral and splanchnic circulations. However, Ang Ⅱ levels were significantly reduced in the splanchnic circulation when compared to the peripheral circulation ($P < 0.05$, Table 3). The ratios between angiotensins

Table 3 Peripheral and splanchnic RAS components during the pre-anhepatic stage of liver transplantation

RAS components	Peripheral measurements	Splanchnic measurements
PRA (ng Ang I/mL per hour)	2.4 ± 0.6	2.5 ± 0.4
Ang I (pg/mL)	764 ± 115	765 ± 103
Ang II (pg/mL)	138 ± 23	97 ± 13[a]
Ang-(1-7) (pg/mL)	48 ± 5	48 ± 7

Data are expressed as mean ± SE. [a]$P < 0.05$ for the comparison between peripheral and splanchnic measurements (paired Student *t*-test).

were also different in the peripheral and splanchnic circulation (Figure 2). The Ang-(1-7)/Ang Ⅱ ratio was higher in the splanchnic circulation than in the peripheral circulation (0.52 ± 0.08 *vs* 0.38 ± 0.04, $P < 0.05$, Figure 2), whereas the peripheral circulating Ang Ⅱ/Ang Ⅰ ratio was elevated in comparison to splanchnic levels (0.18 ± 0.02 *vs* 0.13 ± 0.02, $P < 0.05$, Figure 2). No differences were detected in the Ang-(1-7)/Ang Ⅰ ratio between both sites (Figure 2).

Correlation between hemodynamic parameters and RAS profile during the pre-anhepatic stage of liver transplantation

In general, hemodynamic parameters from patients with cirrhosis (LT group) were different from the reference values. Systemic vascular resistance (555 ± 57 dyn.s.cm^{-5} *vs* 1200-1500 dyn.s.cm^{-5}) and respective index (1029 ± 95 dyn.s.cm^{-5}.m^{-2} *vs* 2400-2900 dyn.s.cm^{-5}.m^{-2}) were below the reference range, whereas cardiac output (9.5 ± 1.1 L/min *vs* 3.0-7.0 L/min) and its index (5.0 ± 0.5 L.min^{-1}.m^{-2} *vs* 2.5-4.0 L.min^{-1}.m^{-2}) were above the reference range. There was no significant correlation between Ang-(1-7) or Ang Ⅱ concentrations and hemodynamic parameters. However, the Ang-(1-7)/Ang Ⅱ ratio was positively correlated with cardiac output ($r = 0.66$, $P < 0.05$) and negatively correlated with systemic vascular resistance ($r = -0.70$, $P < 0.05$) (Figure 3).

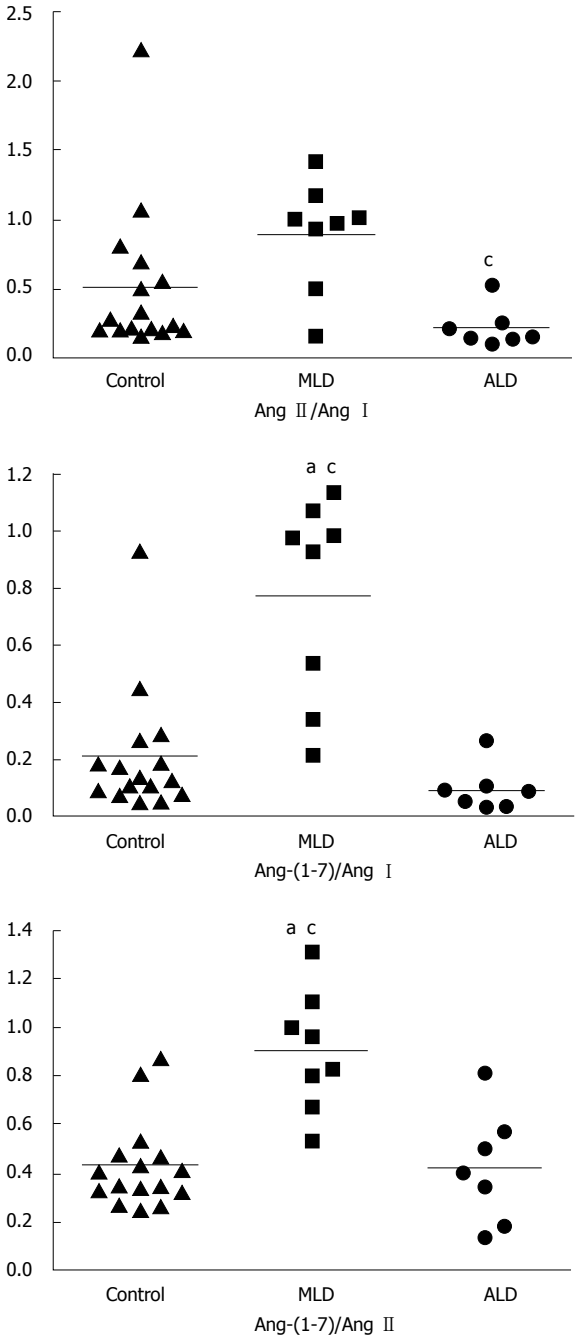

Figure 1 Ratios between angiotensins in healthy controls, and in patients with mild-to-moderate (MLD) and advanced liver disease (ALD). [a]$P < 0.05$ for the comparisons with the control group (Kruskal-Wallis followed by Dunn test for median comparisons). [c]$P < 0.05$ for the comparison between the MLD and ALD groups (Kruskal-Wallis followed by the Dunn test for median comparisons). Ang Ⅰ: Angiotensin Ⅰ; Ang Ⅱ: Angiotensin Ⅱ; Ang-(1-7): Angiotensin-(1-7).

DISCUSSION

The present study supports the concept that RAS may contribute to circulatory dysfunction in human cirrhosis. In general, our data showed that the progression of liver dysfunction is characterized by marked changes in circulating Ang-(1-7) and Ang Ⅱ levels. In the initial stages, the circulating RAS is not activated, although there is a predominance of Ang-(1-7), the vasodilator, rather than Ang Ⅱ. On the other hand, the advanced stages of cirrhosis show an activation of peripheral and splanchnic RAS, and a metabolic deviation toward the RAS vasodilator axis in the splanchnic circulation. Furthermore, we observed a positive correlation between the Ang-(1-7)/Ang Ⅱ ratio and cardiac output as well as a negative correlation between this ratio and systemic vascular resistance, indicating that the final functional effects of the RAS may reflect a balance between these 2 opposing axes. Taken together, these findings suggest a dynamic change in RAS profile according to disease stage (mild-to-moderate *vs* advanced) and vascular bed (peripheral *vs* splanchnic circulation), which could interfere with hemodynamic parameters in human cirrhosis.

The first part of this study evaluated circulating RAS components according to the stage of liver disease and without the interference of any kind of RAS blockade. The RAS profile was completely different in mild-to-moderate cirrhosis when compared to advanced liver disease. The upstream RAS components (PRA and Ang Ⅰ), which are common to both RAS axes and indicate

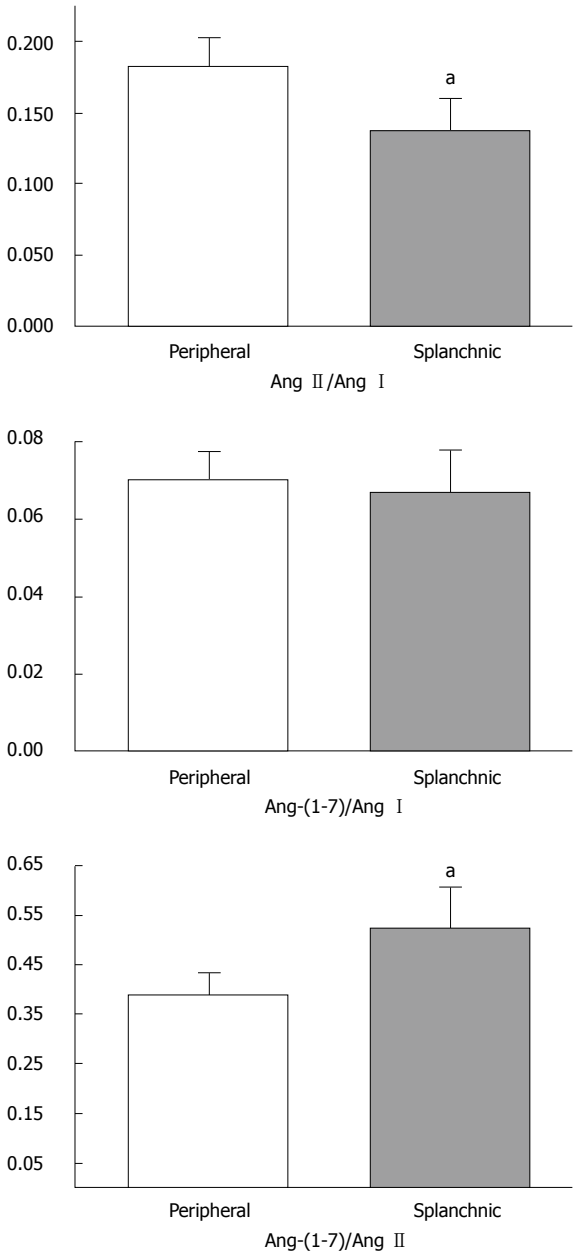

Figure 2 Ratios between angiotensins in the peripheral and splanchnic circulations of liver transplanted patients during the pre-anhepatic stage of liver transplantation. [a]*P* < 0.05 for the comparison between the peripheral and splanchnic ratios (paired Student *t*-test). Ang Ⅰ: Angiotensin Ⅰ; Ang Ⅱ: Angiotensin Ⅱ; Ang-(1-7): Angiotensin-(1-7).

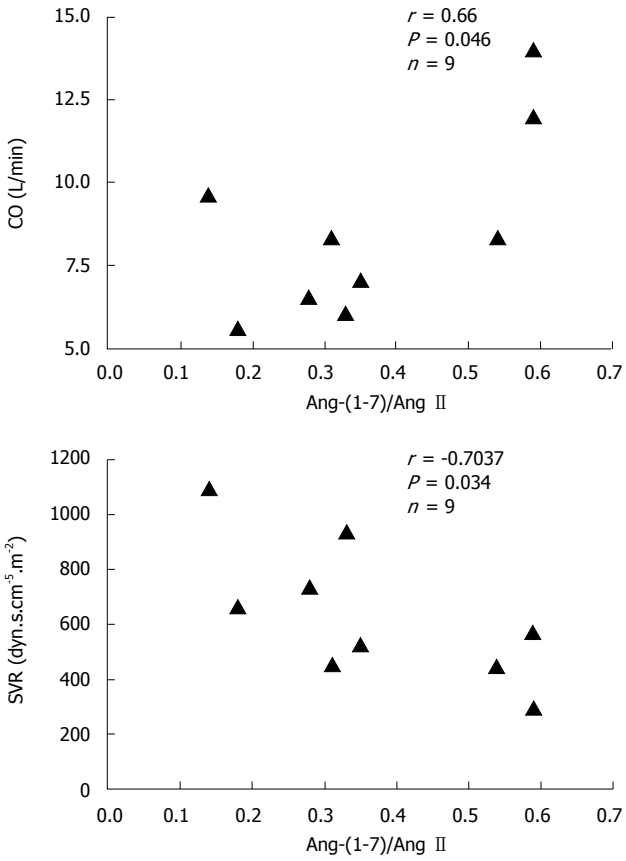

Figure 3 Correlations between Ang-(1-7)/Ang Ⅱ ratios and CO and SVR. Correlation coefficients (*r*) and *P* values were calculated by Spearman's test.

the level of system activation, were increased in ALD patients compared to MLD and healthy controls. Ang Ⅰ and Ang Ⅱ levels were also reduced in the MDL group even in comparison to healthy controls, whereas the ALD group exhibited an overall elevation of PRA and angiotensins when compared to other groups. Other studies have also detected increased PRA and Ang Ⅱ levels in ALD patients[8,18]. In the initial stages of human cirrhosis, PRA measurements revealed a non-activated or even suppressed circulating RAS[16,18]. In this regard, we have recently found that chronic treatment with propranolol in advanced cirrhotic patients is characterized by marked changes in the precursors of the RAS cascade (renin and Ang Ⅰ) with repercussions in the 2 main components of the RAS [Ang Ⅱ and Ang-(1-7)] in the splanchnic and peripheral circulation[15]. Additionally, treatment with propranolol seemed to be able to control the hyperdynamic circulation of cirrhotic patients probably resulting from an overall RAS inhibition, but without changes in the balance between the 2 RAS arms: ACE-Ang-AT1 receptor (vasoconstrictor) *vs* ACE2-Ang-(1-7)-Mas receptor (vasodilator)[15]. Taken together, our previous and present data further support the idea that a possible therapeutic approach for advanced human cirrhosis could be the combination of beta blockade with AT_1 receptor antagonism or ACE inhibition.

Experimental studies have recently evaluated Ang-(1-7) in bile duct-ligated (BDL) rats. Paizis *et al*[14] demonstrated that in both BDL rat and human livers there was an increased ACE2 expression and activity that might facilitate the conversion of Ang Ⅱ to Ang-(1-7). Pereira *et al*[12] demonstrated that the progression of liver dysfunction in BDL rats was characterized by marked changes in Ang-(1-7) and Ang Ⅱ levels and the overall activation of circulating RAS was associated with the progression of hepatic fibrosis. Subsequently, Herath *et al*[13] also found RAS activation in chronic liver injury to be associated with upregulation of ACE2, Mas receptor and hepatic conversion of Ang Ⅱ to Ang-(1-7), leading to increased circulating Ang-(1-7). Taken together, these studies support the presence of an activated ACE2-Ang-(1-7)-Mas receptor axis in liver injury that may counteract the effects of Ang Ⅱ[12,13]. Indeed, blockade of Ang-(1-7) with A-779 worsened the cirrhosis evolution in BDL rats by increasing

liver fibrosis[12].

As mentioned above, in the circulation, RAS seems to act through 2 opposing axes, the classic ACE-Ang Ⅱ-AT1 receptor (vasoconstrictor) and the counter-regulatory ACE2-Ang-(1-7)-Mas receptor (vasodilator)[1,2]. The increase of the Ang-(1-7)/Ang Ⅱ ratio suggests a deviation of RAS metabolism toward Ang-(1-7) formation in MLD patients when compared to the ALD group. The comparison between peripheral and splanchnic levels of angiotensins also revealed a differential regulation in LT patients. While an increased Ang-(1-7)/Ang Ⅱ ratio was detected in the splanchnic circulation, the ratio between Ang Ⅱ and Ang Ⅰ (an indirect estimation of net ACE activity) predominated in the peripheral circulation. Arterial splanchnic vasodilation was consistently detected in human cirrhosis[8,19]. A large number of studies have demonstrated the vasodilatory effect of Ang-(1-7)[20-23]. Possible mechanisms for this effect include bradykinin potentiation[21,22], enhanced prostacyclin (PGI_2) release from vascular smooth muscle cells[23] and direct stimulation of nitric oxide (NO) synthesis[20,22]. NO, PGI_2, carbon monoxide, endocannabinoids and other vasodilators have been associated with arterial splanchnic vasodilation[24]. Our results suggest that the reduction of Ang Ⅱ levels accompanied by an increased Ang-(1-7)/Ang Ⅱ ratio in the splanchnic circulation may be, at least in part, responsible for changes in vascular splanchnic tone. A possible explanation for this finding is that the elevated expression and activity of ACE2[13,14] during hepatic injury could promote the synthesis of Ang-(1-7). During initial stages of the disease, hyperdynamic circulation is responsible for the maintenance of cardiovascular system homeostasis. The increase of the Ang-(1-7)/Ang Ⅱ ratio in the peripheral circulation in MLD patients suggests that a deviation of RAS metabolism toward the vasodilator axis could be responsible at least in part for the reduction of the systemic vascular resistance. However, as the disease progresses, the splanchnic vasodilation continues to increase and the hyperdynamic circulation is insufficient to compensate for the effective arterial hypovolemia, leading to a reduction in blood pressure, which, in turn, could stimulate the classic ACE-Ang Ⅱ-AT_1 receptor axis of the RAS in the peripheral circulation, the sympathetic nervous system and vasopressin release, as an attempt to restore blood volume and systemic perfusion pressure[9]. At this stage, in spite of the continued increase in splanchnic vasodilation, the systemic vascular resistance is kept low although constant. As the splanchnic circulation is resistant to the effect of Ang Ⅱ, noradrenaline and vasopressin due to the local release of NO and other vasodilators[25], the maintenance of arterial pressure could be the result of vasoconstriction in extra-splanchnic vascular areas[26]. This is in accordance with the reduction in the Ang-(1-7)/Ang Ⅱ ratio in the peripheral circulation from mild-to-moderate to advanced stages of cirrhosis, and from the splanchnic to the peripheral circulation in LT patients.

The correlation between the Ang-(1-7)/Ang Ⅱ ratio and hemodynamic parameters (cardiac output and systemic vascular resistance) suggests that not only Ang Ⅱ, but also an excessive formation of Ang-(1-7) may be involved in circulatory changes in human cirrhosis. Cardiac output and systemic vascular resistance depends on the balance between the 2 RAS axes. Besides vascular effects, the literature has also provided evidence of a role for Ang-(1-7) in the regulation of cardiac function[27,28]. Indeed, Ang-(1-7) increases cardiac output and stroke volume in rats[28]. Moreover, the finding that isolated hearts from Mas-knockout mice exhibited an impaired function and an increase in coronary vascular resistance supports the importance of the Ang-(1-7)-Mas receptor axis in cardiovascular function[28]. In summary, the cardiovascular effects of Ang-(1-7) suggest a further mechanism by which RAS may contribute to altered vascular tone in cirrhosis.

It should be also pointed that we are aware of the limitations of our study design. First, peripheral blood samples generally represent the cumulative expression of RAS in multiple tissues and may not reliably reflect molecular activity in the splanchnic circulation. For this reason, we did manage to collect samples from the portal vein during liver transplantation. However, it is still difficult to compare these findings to the samples collected in the peripheral blood from outpatients. Another concern is the use of diuretics in ALD patients which could produce relative hypovolemia and activation of the ACE-Ang Ⅱ-AT_1 receptor axis. However, the diuretics are probably not the sole cause of this activation, since their interruption normally reduces but does not normalize PRA[29]. Nevertheless, some aspects of this study may increase the strength of our findings, such as the utilization of strictly defined inclusion and exclusion criteria and the well-established protocol for the measurements of PRA and angiotensins.

In conclusion, based on our previous[15] and present findings, the relationship of Ang-(1-7) and Ang Ⅱ may play a role in hemodynamic changes of human cirrhosis. We hypothesize that the ACE2-Ang-(1-7)-Mas receptor axis predominates in the peripheral circulation in the initial stages of disease (MLD patients) and in the splanchnic circulation in the advanced stages of cirrhosis (LT group), both contributing to a reduction in vascular resistance and consequently to hyperdynamic circulation. In the peripheral circulation of ALD patients, when compared to the splanchnic circulation, the ACE-Ang Ⅱ-AT_1 receptor arm predominates, probably leading to extra-splanchnic vasoconstriction that occurs at this stage. However, further studies with a larger number of patients should address the precise role of RAS in human cirrhosis. If these preliminary data are confirmed, future therapies interfering with 2 RAS axes in both the systemic and splanchnic circulations should lead to more success in the management of reversible fibrosis[30] and hemodynamic changes in human cirrhosis.

COMMENTS

Background

Liver cirrhosis has been recently studied in the light of the new view of the renin angiotensin system (RAS). It is becoming clear that the RAS can influence liver cirrhosis through its 2 main arms. While the angiotensin converting enzyme (ACE)-angiotensin (Ang) Ⅱ-AT1 receptor arm contributes to liver tissue injury and fibrosis and the maintenance of basal vascular tonus in non-compensated cir-

rhosis, the activation of the ACE2-Ang-(1-7)-Mas receptor arm exerts anti-fibrotic actions and probably has also a role in arterial vasodilation in liver cirrhosis.

Research frontiers

This study represents an initial approach to understand how RAS mediators change according to the stage of human cirrhosis and, more importantly, how the relationship between Ang-(1-7) and Ang Ⅱ may affect the hemodynamic parameters during liver transplantation.

Innovations and breakthroughs

The data showed that the progression of liver dysfunction is characterized by marked changes in circulating Ang-(1-7) and Ang Ⅱ levels. At the initial stages, there is a predominance of Ang-(1-7) rather than Ang Ⅱ. On the other hand, advanced stages of cirrhosis show an activation of peripheral and splanchnic RAS, and a deviation toward the formation of Ang-(1-7) in the splanchnic circulation. Furthermore, there was a positive correlation between the Ang-(1-7)/Ang Ⅱ ratio and cardiac output and a negative correlation between this ratio and systemic vascular resistance, indicating that the final functional effects of the RAS may reflect a balance between these 2 opposing peptides.

Applications

According to this study, future therapies modifying the 2 RAS axes in both the systemic and splanchnic circulation should lead to more success in the management of reversible fibrosis and the hemodynamic changes in human cirrhosis.

Peer review

The manuscript "The relationship between angiotensin (1-7) and angiotensin Ⅱ correlates with hemodynamic changes in human liver cirrhosis" is a preliminary, well designed study with a small cohort of patients for each cirrhotic group studied, presented by an experienced research group that, on the basis of the results of this study and those obtained in a previous work (published in World J Gastroenterol, 2008), concluded that the relationship between Ang-(1-7) and Ang Ⅱ may play a role in hemodynamic changes in human cirrhosis. If the results are corroborated in future studies, a novel therapy could be designed for management of reversible fibrosis and hemodynamic changes in human cirrhosis.

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BRIEF ARTICLES

Checkpoint with forkhead-associated and ring finger promoter hypermethylation correlates with microsatellite instability in gastric cancer

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Abstract

AIM: To examine the methylation status of the promoter region of the checkpoint with forkhead-associated and ring finger (CHFR) and microsatellite mutator status in 59 primary gastric cancers.

METHODS: We investigated the promoter methylation of CHFR in 59 cases of gastric cancer using methylation-specific PCR. Five microsatellite loci were analyzed using high-intensity microsatellite analysis reported previously, and *p53* gene mutations were investigated by direct sequencing.

RESULTS: Twenty cases (33.9%) showed promoter methylation and no relation was observed with the clinicopathological factors. We found that the promoter methylation of CHFR was frequently accompanied with microsatellite instability (MIN). Seven of 20 (35.0%) cases showed MIN in hypermethylation of the CHFR tumor, while three of 39 (7.7%) cases showed MIN in the non-methylated CHFR tumor ($P < 0.01$). However, we failed to find any relationship between CHFR methylation and p53 mutation status.

CONCLUSION: The coordinated loss of both the mitotic check point function and mismatch repair system suggests the potential to overcome the cell cycle check point, which may lead to an accumulation of mutations. However, the *p53* mutation was not related to hypermethylation of the CHFR promoter and MIN, which indicates that an abnormality in p53 occurs as an independent process from the mismatch repair deficiency in carcinogenesis.

Key words: Checkpoint with forkhead-associated and ring finger; Methylation; Microsatellite instability; Gastric cancer; p53

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INTRODUCTION

Checkpoint with forkhead-associated and ring finger (CHFR) is a recently identified gene, which is localized to chromosome 12q24.33[1]. CHFR functions as an important checkpoint protein early in the G2/M transition and its activation delays the cell cycle in prophase, thus preventing chromosome condensation in response to the mitotic stress induced by nocodazole or paclitaxel[2-4]. In addition, CHFR promotes cell survival in response to mitotic stress. CHFR is ubiquitously expressed in normal tissues; however, it is frequently down-regulated in human cancer, mostly as a result of hypermethylation of its CpG island in the promoter region. CHFR down-regulation has been found in primary lung, colon, esophagus, nasopharyngeal and gastric carcinomas[5-12]. In gastric cancer, CHFR

promoter hypermethylation has been reported to lead to chromosome instability[13]. Another study has shown that the aberrant methylation of CHFR appears to be a good molecular marker with which to predict the sensitivity of gastric cancer to microtubule inhibitors[4]. In this study, we first investigated and showed CHFR methylation and microsatellite instability in gastric cancer patients.

Genetic instability is one of the hallmarks of human cancer. In colon cancer, tumors with chromosomal instability (CIN) can be distinguished from those with microsatellite instability (MIN), thereby showing instability in the GC-rich tandem repeat[14,15]. While the former frequently show aneuploidy, the karyotype in the latter is usually preserved. In gastric cancer, tumors with CIN are frequently observed and such cancers show poor prognosis and p53 mutation, such as colon cancer[16-18]. However, whether gastric cancer can be categorized into CIN and MIN phenotypes like colon cancer remains to be elucidated. An aberrant CHFR function leads to the disruption of normal chromosomal segregation, and it could be considered as a cause of CIN. However, a recent study failed to show any correlation between CIN and the loss of CHFR function[19], although CHFR knockout mice show CIN[20]. On the other hand, the MIN phenotype has been shown to be associated with the hypermethylation of the CHFR promoter[19,21,22]. The hypermethylation of CHFR and hMLH1 has been shown to occur concurrently, and CHFR methylation is not associated with CIN in gastric cancer[21].

We have previously reported the methylation status of the promoter region of the CHFR gene in 110 primary breast cancers[5]. We observed hypermethylation of the CHFR promoter region in only one case (0.9%) of breast cancer. Intriguingly, the only case that revealed the hypermethylation of the CHFR promoter region also showed the MIN phenotype. In the present study, we examined the methylation status of the promoter region of CHFR and microsatellite mutator status in 63 primary gastric cancers. This is believed to be the first study to show the striking relationship between CHFR silencing and MIN in gastric cancers.

MATERIALS AND METHODS

Specimens and extraction of genomic DNA

Fifty-nine primary gastric carcinomas and paired normal tissue specimens were obtained from Japanese patients who underwent surgery at the Department of Surgery and Science, Kyushu University Hospital, from 1999 to 2002. Informed consent was obtained from all patients prior to tissue acquisition. Immediately after resection, the specimens were placed in liquid nitrogen and then were used for analysis of genomic DNA. The remaining tissue specimens were routinely processed for histopathological analysis by histopathology specialists in our hospital. The histopathological diagnosis was determined according to the criteria of the Japanese Gastric Cancer Society. Frozen tissue specimens were broken up in liquid nitrogen and lysed in digestion buffer (10 mmol/L Tris-HCl, pH 8.0, 0.1 mol/L EDTA, pH 8.0, 0.5% SDS, 20 μg/mL pancreatic RNase). After treatment with proteinase K and extraction with phenol, DNA was precipitated with ethanol, and then was dissolved in 1 TE (10 mmol/L Tris-Cl; pH 7.5, 1 mmol/L EDTA).

Methylation analysis

Sodium bisulfite conversion of genomic DNA was performed using the EZ DNA Methylation Kit (Zymo Research, Orange, CA, USA), which integrates DNA denaturation and bisulfite conversion processes into a single step, followed by rapid in-column desulfonation and DNA clean-up, according to the manufacturer's instructions. Methylation-specific PCR (MS-PCR) was carried out with the following oligonucleotide primers, which were designed to be specific to either methylated or unmethylated DNA after sodium bisulfite conversion as described above. Methylated DNA-specific primers were MF1 (forward: 5'-ATATAATATGGCGTCGATC) and MR1 (reverse: 5'-TCAACTAATCCGCGAAACG). Unmethylated DNA-specific primers were UF1 (forward: 5'-ATATAATATGGTGTTGATT) and UR1 (reverse: 5'-TCAACTAATCCACAAAACA)[18]. PCR amplification consisted of 35 cycles of 94℃ for 1 min, 58℃ for 1 min, and 72℃ for 1 min (MF1 and MR1); and 94℃ for 1 min, 48℃ for 1 min and 72℃ for 1 min (UF1 and UR1). The resultant PCR products were separated on 2% agarose gels. CpGenome Universal Methylated DNA (Chemicon International, Temecula, CA, USA), which is enzymatically methylated human male genomic DNA, was used as a positive control for MS-PCR. Purified genomic DNA isolated from the human placenta (BioChain Institute, Hayward, CA, USA) was used as a negative control for non-methylated DNA. All analyses included positive and negative controls, and were performed at least twice.

MIN analysis

Five human dinucleotide microsatellites, D2S123, D5S107, D10S197, D11S904 and D13S175, were used as a marker for the MIN analysis. Using genomic DNA derived from the tissue specimens, the five microsatellite sequences were amplified by PCR. The oligonucleotide primers that corresponded to the microsatellite sequences were synthesized and purified by HPLC, and the forward primers were labeled with fluorescent compounds, ROX (6-carboxy-x-rhodamine), 6-FAM (6-carboxyfluorescein) or HEX (6-carboxy-2',4',7',4,7,-hexachloro-fluorescein). PCR reactions were performed using Tamara Taq Reagent Kits (Takara Bio, Ohtsu, Japan) and Applied Biosystems GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA, USA). A 50-mL reaction mixture contained 1 × reaction buffer, 350 mmol/L each dNTP, 10 pmol each primer, 2.5 U Taq polymerase and 25 ng genomic DNA. The thermal conditions of the system were as follows: one cycle at 95℃ for 4 min; 35 cycles at 95℃ for 30 s, 55℃ for 30 s, and 72℃ for 30 s; and one cycle at 72℃ for 10 min. Next, 0.5 U T4 DNA polymerase was added to the mixture, followed by incubation at 37℃ for 10 min. To compare electrophoretic profiles between two samples, 6-FAM- or ROX-labeled products and HEX-labeled products were mixed, denatured and loaded onto an ABI

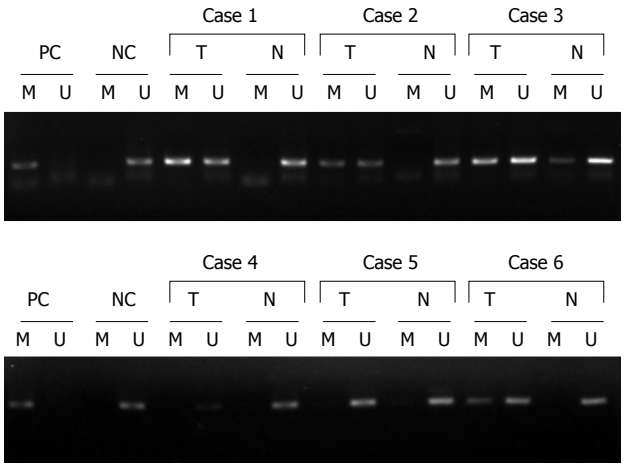

Figure 1 Methylation of the promoter region of the *CHFR* gene in primary gastric cancer, analyzed by MS-PCR. The U lane represents the amplification of unmethylated alleles, while the M lane represents that of methylated alleles. CHFR methylation was present in cases 1, 2, 3 and 6. CpGenome Universal Methylated DNA, which is enzymatically methylated human male genomic DNA, was used as a positive control (PC), and purified genomic DNA isolated from human placenta was used as a negative control (NC) as non-methylated DNA. T: Tumor; N: Normal.

373A DNA Sequencer or ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The data were processed using the ABI software program, GeneScan ver. 1.2.2. or 3.1.2 (Applied Biosystems).

DNA sequence analysis of p53

The base sequence was determined from exon 4 to 8 of p53 using a PCR direct sequence. The PCR product of p53 was purified with a Microcon-100 Microconcentrator (Amicon, Beverly, MA, USA). The direct sequencing of PCR products was performed using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Norwalk, CT, USA). The cycle sequence product was electrophoresed and analyzed on the Applied Biosystems Model 311 Genetic Analyzer (Perkin-Elmer).

RESULTS

CHFR promoter hypermethylation in gastric cancer

We studied the methylation status of CHFR for 59 cases of primary gastric cancer. We detected the hypermethylation of the CHFR promoter in 20 of 59 (33.9%) cancer tissue specimens studied (Figure 1 and Table 1), but only in 6 of 59 (10.2%) of the corresponding non-neoplastic gastric mucosa specimens (Table 1). CHFR hypermethylation occurred at a similar frequency in early and advanced gastric cancers, and none of the clinicopathological factors correlated with hypermethylation of the CHFR promoter (Table 2). The methylation status did not significantly influence the event-free survival rate as analyzed by a Kaplan-Meier curve (data not shown).

Table 1 Summary of gastric cancer and methylation of CHFR

Case No.	Age (yr)	Sex	Lauren classification	CHFR methylation		MIN status[2]	P53 mutation
				Tumor	Normal		
1	86	M	Intestinal	+	-	MSS	Mutant
2	67	F	Diffuse	+	-	MSI-L	Wild
3	76	F	Intestinal	+	+	MSS	Wild
4	30	F	Diffuse	-	-	MSS	Wild
5	74	M	Diffuse	-	+	ND[1]	Mutant
6	42	M	Diffuse	+	+	MSS	Wild
7	78	M	Diffuse	-	-	MSS	Wild
8	55	M	Diffuse	-	-	MSS	Mutant
9	50	F	Diffuse	-	-	MSS	Wild
10	55	M	Intestinal	-	-	MSS	Wild
11	63	M	Diffuse	-	-	MSS	Wild
12	69	M	Intestinal	-	+	MSS	Mutant
13	52	M	Intestinal	-	-	MSS	Wild
14	80	M	Diffuse	+	-	MSI-H	Wild
15	73	F	Diffuse	-	-	MSS	Wild
16	74	M	Intestinal	-	-	MSS	Wild
17	39	F	Diffuse	-	-	MSS	Wild
18	53	F	Diffuse	+	-	MSS	Wild
19	59	M	Diffuse	+	-	MSS	Mutant
20	72	M	Intestinal	+	-	MSS	Wild
21	69	M	Intestinal	-	-	MSS	Wild
22	51	M	Diffuse	-	-	MSS	Wild
23	61	M	Diffuse	-	-	MSI-L	Wild
24	65	M	Diffuse	-	-	MSI-L	Wild
25	56	M	Diffuse	-	-	MSS	Wild
26	53	M	Diffuse	-	-	MSS	Wild
27	42	M	Diffuse	-	-	MSI-H	Wild
28	59	F	Intestinal	-	-	MSS	Mutant
29	67	M	Intestinal	-	-	MSS	Mutant
30	55	F	Diffuse	-	-	MSS	Wild
31	68	M	Intestinal	+	-	MSI-L	Wild
32	73	M	Intestinal	-	-	MSS	Wild
33	49	F	Diffuse	+	-	MSS	Wild
34	52	F	Diffuse	-	-	MSS	Wild
35	69	M	Diffuse	-	-	MSS	Wild
36	73	M	Intestinal	+	-	MSS	Wild
37	63	M	Diffuse	-	-	MSS	Wild
38	73	F	Intestinal	-	-	MSS	Wild
39	65	M	Intestinal	-	-	MSS	Wild
40	60	M	Diffuse	-	-	MSS	Mutant
41	48	F	Diffuse	-	-	MSS	Wild
42	69	F	Diffuse	+	-	MSI-L	Wild
43	81	F	Intestinal	+	-	MSS	Wild
44	60	M	Intestinal	+	+	MSI-L	Wild
45	59	M	Intestinal	+	-	MSI-H	Wild
46	57	M	Diffuse	+	-	MSS	Wild
47	66	F	Intestinal	-	-	MSS	Wild
48	68	M	Intestinal	-	-	MSS	Wild
49	56	F	Intestinal	-	-	MSS	Wild
50	64	F	Diffuse	+	-	MSS	Wild
51	45	F	Diffuse	-	-	MSS	Wild
52	68	M	Diffuse	-	-	MSS	Wild
53	67	M	Diffuse	-	-	MSS	Wild
54	66	M	Diffuse	-	-	ND	Wild
55	55	M	Diffuse	+	-	MSI-H	Wild
56	52	F	Intestinal	+	-	MSS	Wild
57	77	F	Intestinal	+	-	MSS	Wild
58	61	M	Intestinal	-	+	ND	Wild
59	70	F	Diffuse	-	-	MSS	Wild

[1]ND: Not detected; [2]MSI-H: High level of miscrosatellite instability; MSI-L: Low level of microsatellite instability; MSS: Microsatellite stability.

Microsatellite alterations

MIN was analyzed and we confirmed the results for 56 of 59 samples. MIN was recognized in 10 of 56 cases (17.2%) (Table 2). We observed a strong correlation be-

Table 2 Clinicopathological features of gastric cancer and methylation of CHFR

Variable	Negative *n* = 39	Methylated *n* = 20	*P* value
Gender			
Male	26	11	NS
Female	13	9	
Age	60.5	64.6	NS
Histology			
Intestinal	14	10	NS
Diffuse	25	10	
Serosal invasion			
Negative	15	8	NS
Positive	24	12	
Histological lymph node metastasis			
Negative	15	5	NS
Positive	24	15	
Vascular involvement			
Negative	22	14	NS
Positive	14	6	
Peritoneal dissemination			
Negative	32	17	NS
Positive	7	3	
Stage			
Ⅰ + Ⅱ	12	5	NS
Ⅲ + Ⅳ	27	15	
p53 mutation			
Wild	33	18	NS
Mutation	6	2	
MIN status			
MSI-H/L	3	7	< 0.01
MSS	36	13	

NS: Not significant.

tween hypermethylation of CHFR and MIN. Seven of 20 (35.0%) cases showed MIN in hypermethylation of the CHFR tumor, while three of 39 (7.7%) cases showed MIN in the non-methylated CHFR tumor.

p53 mutation

All 59 of the gastric cancer cases were investigated for a mutation in exons 5-9 of p53. The mutation spectrum and the discussion about the rate of frequency have all been previously reported[23]. We detected eight mutations of p53, however, we failed to find any relationship between CHFR methylation and p53 mutation status.

DISCUSSION

It has been proposed that the spontaneous mutation rate in normal cells is not sufficient to generate the number of mutations found in human cancers, since there are large numbers of mutations observed in human cancers[24]. In other words, cancer cells exhibit genetic instability. It is known that there are two types of genetic instability in gastrointestinal cancer carcinogenesis, CIN and MIN[14,15].

In colon cancer, tumors with CIN can be clearly distinguished from those with MIN. While the former frequently show aneuploidy, the karyotype in the latter is usually preserved. The MIN tumors, as a result of a defect in DNA mismatch repair, show instability in the GC-rich tandem repeat, the so-called MIN, which is interspersed into the genome. The DNA mismatch repair MMR system as represented by hMLH1 is essential for maintaining genomic stability and preventing tumor formation, and it is highly conserved in evolution. Recent studies have shown that MMR proteins are required for the S-phase checkpoint activation induced by ionizing irradiation[25], and the G2-checkpoint activation induced by cisplatin, SN1 DNA methylators, and 6-thioguanine[26,27].

In gastric cancer, tumors with CIN have been frequently observed and such cancers show a poor prognosis, and *p53* mutation[16-18]. However, gastric cancer has not been clearly categorized into CIN and MIN phenotypes, such as for colon cancer, since gastric cancer has various types of histological groups, and MIN tumors do not occur as frequently as in colon cancer[28]. CHFR is a recently identified gene, which functions as an important checkpoint protein early in the G2/M transition, and its activation delays the cell cycle in prophase, thus preventing chromosome condensation in response to mitotic stress[1]. The aberrant CHFR function leads to the disruption of normal chromosomal segregation, and it could thus be considered as a cause of CIN. However, a recent study has failed to show any correlation between CIN and the loss of CHFR function[19]. In contrast to the mismatch repair genes, CHFR does not seem to participate in the DNA damage checkpoint or DNA repair pathways. CHFR regulates an early mitotic checkpoint, during prophase, in response to the disruption of normal microtubule formation or stabilization, as assessed after treatment with microtubule poisons such as nocodazole, colcemid and taxanes[1]. Recently, the association between the hypermethylation of the *MMR* gene of hMLH1 promoter and that of the CHFR promoter has been reported[22]. Brandes *et al*[22] have reported a correlation between hMLH1 and CHFR methylation in cell lines with the MIN phenotype in colon cancer. They have reported that there is no correlation between promoter methylation of CHFR and other genes, including those that have been shown to be silenced by promoter methylation in the CIMP (CpG island methylation) phenotype. These results have suggested that a relationship exists between CHFR methylation and the MIN phenotype, but not the CIN phenotype. Along with this suggestion, our results show a direct relationship between the MIN phenotype and the promoter methylation status of CHFR in gastric cancer. We previously reported the methylation status of the promoter region of the CHFR gene in 110 primary breast cancers[5]. We observed the hypermethylation of the CHFR promoter region in only one case (0.9%). Intriguingly, only the one case that revealed hypermethylation of the CHFR promoter region showed the MIN phenotype. These results show the direct relationship between MIN and CHFR promoter methylation.

The majority of gastric cancers exhibit DNA aneuploidy[16-18]. It is presumed that unknown genetic defects lead to CIN, although no such abnormalities which are directly associated with CIN have been identified. CHFR

is a possible inducer of CIN, however, CHFR abnormality associated with CIN has not been demonstrated. Rather, CHFR methylation was found in the case of breast cancer with the MIN tumor. The significant correlation between methylation of CHFR and MIN suggests that the loss of CHFR expression allows the cells, which are deficient in MMR activity, to progress through the G2/M cell cycle checkpoint without delay. MMR genes are also important in the regulation of the G2/M checkpoint. Our previous study has shown the importance of MMR genes at the G2/M arrest point in the response against 5-fluorouracil (5-FU)[29]. The normal p53 cell line underwent both G1 and G2/M arrest after treatment with 5-FU. The cell line with mutated *p53* failed to undergo G1 arrest but showed G2/M arrest. The cell lacking the *MMR* gene failed to undergo G2/M arrest but underwent G1 arrest. These results shows that *MMR* genes are associated with G2/M arrest. It has been reported that promoter methylation is an early event in the process of carcinogenesis, as extensive methylation is found in the colon polyp. Therefore, abnormality of both the CHFR and MMR systems provides a survival advantage for gene alterations in carcinogenesis, since the cell cycle does not stop at the G2/M checkpoint without the CHFR and MMR system, even if there is DNA mismatch or damage. This is thought to be one of the mechanisms that generate a mutator phenotype in cancer. However, in our study, the *p53* mutation was not frequent in cases that showed methylation of CHFR and MIN. Usually, both a *p53* mutation and loss of heterozygosity of *p53* are observed in CIN tumors. *p53* mutation has been found only rarely in tumors that show MIN, and this is evidence for the presence of two different pathways for colon carcinogenesis.

In conclusion, we herein demonstrated a correlation between the hypermethylation of CHFR and the MIN of gastric cancer patients. Both MIN and CHFR hypermethylation induce mitotic check point disruption and confer a survival advantage to the cells, however, this survival advantage does not lead to either *p53* mutation or CIN in gastric cancer.

COMMENTS

Background

Checkpoint with forkhead and ring finger (CHFR) is a mitotic stress checkpoint gene whose promoter is frequently methylated in various kinds of cancer. In this study, the authors examined the methylation status of the promoter region of CHFR and microsatellite mutator status in 59 primary gastric cancers.

Research frontiers

An aberrant CHFR function leads to the disruption of normal chromosomal segregation, and it could thus be considered as a cause of chromosomal instability (CIN). However, a recent study failed to show any correlation between CIN and the loss of CHFR function, although CHFR knockout mice did show CIN.

Innovations and breakthroughs

This is believed to be the first study to show the striking relationship between CHFR silencing and microsatellite alteration in gastric cancer.

Applications

The results suggest that an abnormality in the CHFR and MMR systems may provide a survival advantage in gastric carcinogenesis.

Terminology

CHFR is localized to chromosome 12q24.33. CHFR functions as an important checkpoint protein early in the G2/M transition, and its activation delays the cell cycle in the prophase.

Peer review

This study demonstrated a clear correlation between the hypermethylation of CHFR and the microsatellite instability of gastric cancer patients. As a result, the reviewer think that this is an important paper that contributes positively to progress in this field.

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BRIEF ARTICLES

Risk factors for sporadic colorectal cancer in southern Chinese

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Abstract

AIM: To investigate the role of smoking, alcohol drinking, family history of cancer, and body mass index (BMI) in sporadic colorectal cancer in southern Chinese.

METHODS: A hospital-based case-control study was conducted from July 2002 to December 2008. There were 706 cases and 723 controls with their sex and age (within 5 years) matched. An unconditional logistic regression model was used to analyze the association between smoking, alcohol drinking, family history of cancer, BMI and sporadic colorectal cancer.

RESULTS: No positive association was observed between smoking status and sporadic colorectal cancer risk. Compared with the non alcohol drinkers, the current and former alcohol drinkers had an increased risk of developing sporadic colorectal cancer (CRC) (adjusted OR = 8.61 and 95% CI = 6.15-12.05; adjusted OR = 2.30, 95% CI = 1.27-4.17). Moreover, the increased risk of developing sporadic CRC was significant in those with a positive family history of cancer (adjusted OR = 1.62, 95% CI = 1.12-3.34) and in those with their BMI $\geqslant$ 24.0 kg/m^2 (adjusted OR = 1.39, 95% CI = 1.10-1.75). Stratification analysis showed that the risk of developing both colon and rectal cancers was increased in current alcohol drinkers (adjusted OR = 7.60 and 95% CI = 5.13-11.25; adjusted OR = 7.52 and 95% CI = 5.13-11.01) and in those with their BMI $\geqslant$ 24.0 kg/m^2 (adjusted OR = 1.38 and 95% CI = 1.04-1.83; adjusted OR = 1.35 and 95% CI = 1.02-1.79). The risk of developing colon cancer, but not rectal cancer, was found in former alcohol drinkers and in those with a positive family history of cancer (adjusted OR = 2.51 and 95% CI = 1.24-5.07; adjusted OR = 1.82 and 95% CI = 1.17-2.82).

CONCLUSION: Alcohol drinking, high BMI ($\geqslant$ 24.0 kg/m^2) and positive family history of cancer are the independent risk factors for colorectal cancer in southern Chinese.

Key words: Case-control; Colorectal cancer; Risk factors; Smoking; Alcohol drinking; Body mass index; Family history

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INTRODUCTION

Colorectal cancer (CRC) is one of the most common cancers in China. Although official statistic data are scanty, the reports from some regions indicate that the incidence of colorectal cancer increases[1,2]. During 1974-1999 in Henan Province, the incidence of esophageal carcinoma was significantly decreased

whereas that of CRC has increased over the last two decades[1]. In Guangdong Province, the incidence of CRC in Huidong County is getting close to that in the world[2]. These data suggest that more attention should be paid to the prevention and control of CRC.

In recent years, the epidemiological factors for CRC in Chinese have been extensively studied[3-8]. However, the results are uncertain. It has been reported that smoking is a risk factor for rectal cancer in Chinese[3] and is associated with the increased risk of developing rectal cancer in Singapore Chinese[4]. However, other studies showed that smoking is not a risk factor for CRC in Chinese[5-7]. Similarly, the relation between alcohol drinking and colorectal cancer in Chinese is controversial[3,6,8]. A population-based prospective cohort study showed that alcohol consumption is not significantly associated with the risk of developing CRC[8], but is associated with the risk of developing CRC in northern Chinese[6] and Hong Kong Chinese[3].

The reports about the association between body mass index (BMI) and colorectal cancer in thin Chinese are scanty. A study comprising 931 cases and 1552 controls in Shanghai demonstrated that BMI is a risk factor for colorectal cancer in men and pre-menopausal women[9]. Zhang *et al*[10] reported that family history is positively related with CRC. However, their data lack of multivariate analysis in unconditional logistic regression model adjusted by factors including age, sex, smoking status, alcohol consumption, family history of cancer, and BMI. Thus, the role of epidemiological factors for CRC in Chinese should be further investigated.

MATERIALS AND METHODS

Subjects and data collection

From July 2002 to December 2008, a hospital-based case-control study was conducted in Guangzhou City. Patients with sporadic CRC were recruited in our study with a response rate of about 95%. A total of 513 patients were recruited as a test group between July 2002 and April 2008 at the Sixth Affiliated Hospital (Gastrointestinal and Anal Hospital) of Sun Yat-Sen University (Guangzhou, China), Sun Yat-Sen University Cancer Center (Guangzhou, China), the First Affiliated Hospital of Sun Yat-Sen University (Guangzhou, China) and the Affiliated Tumor Hospital of Guangzhou Medical College (Guangzhou, China). To validate our findings, 193 patients were recruited as a validation group between May 2008 and December 2008 at Guangdong Provincial People's Hospital and Panyu People's Hospital (Guangzhou, China). Cancer-free controls were randomly selected from about 10000 individuals in Guangzhou City during the same period, with a response rate of about 85%. Five hundred and twenty-three controls were recruited as an original test group and 200 controls as a validation group. Cases of familial adenomatous polyposis and those fulfilling the criteria of Amsterdam for hereditary non-polyposis colorectal cancer were excluded. Thus, 706 sporadic CRC patients and 723 cancer-free controls were included in this study. All subjects were genetically-unrelated Han nationality Chinese from Guangzhou City and its surrounding regions. The control subjects were sex and age (within 5 years) matched to the patients. The study was approved by the Review Board of Sun Yat-Sen University.

Exposure assessment

Each participant was scheduled for an interview after he or she gave his or her written informed consent, and a structured questionnaire was designed by the interviewers to collect data on smoking status, alcohol consumption and other factors including BMI, family history of cancer, menstrual history, sex and age. The participants who smoked < 100 cigarettes in their lifetime were defined as non smokers. Otherwise, they were defined as smokers. Smokers who were quitted with smoking for > 1 year prior to enrollment were considered former smokers, and the remaining were defined as current smokers. Similarly, participants who consumed alcohol at least once a week for ⩾ 1 year were defined as alcohol drinkers and the remaining as non alcohol drinkers. Alcohol drinkers who were quitted with drinking for ⩾ 1 year were defined as former alcohol drinkers, and the others as current drinkers. Those with a positive family history of cancer were defined as the first- or second-degree relatives or both. This study used the BMI cutoff points recommended by the Cooperative Meta-Analysis Group on Obesity in China[11]. Subjects with their BMI ⩽ 23.9 kg/m^2 were categorized as underweight and normal body weight, while those with their BMI ⩾ 24.0 kg/m^2 were categorized as overweight and obese.

Statistical analysis

Two-sided chi-square test was performed to assess differences in age, sex, smoking status, alcohol consumption, family history of cancer and BMI between patients and controls. An unconditional logistic regression model was used to estimate the association between case-control status and factors including smoking status, alcohol consumption, BMI, and family history of cancer, measured by odds ratio (OR) and corresponding 95% confidence interval (CI). Logistic regression modeling was used in trend test. Statistical analysis was performed using SPSS for Windows (version 13.0). All statistical analyses were 2-sided and $P < 0.05$ was considered statistically significant.

RESULTS

Characteristics of studied Chinese

A total of 706 sporadic CRC cases and 723 cancer-free controls were included in statistical analysis. The differences in distribution of age, sex, menstrual history between the cases and controls were not statistically significant ($P = 0.992$, 0.937, 0.883, respectively) (Table 1). Compared with the controls, the cases were more likely to be current smokers and current drinkers (current smokers: 44.8% *vs* 30.3%, $P < 0.0001$ and current drinkers: 52.8% *vs* 19.4%, $P < 0.0001$). Moreover, the cases tended to have a higher BMI ($P = 0.002$) and a positive family history of cancer ($P = 0.018$). Therefore, these variables were further

Table 1 Distributions of selected variables in colorectal cancer patients and cancer-free controls *n* (%)

Variables	Test group		Validation group		Merged group		*P*[1]
	Case	Control	Case	Control	Case	Control	
Age (yr)							0.992
≤ 49	120 (23.4)	122 (23.3)	36 (18.7)	36 (18.0)	156 (22.1)	158 (21.9)	
50-60	146 (28.5)	149 (28.5)	39 (20.2)	42 (21.0)	185 (26.2)	191 (26.4)	
> 60	247 (48.2)	252 (48.2)	118 (61.1)	122 (61.0)	365 (51.7)	374 (51.7)	
Sex							0.937
Male	300 (58.5)	300 (57.4)	137 (71.0)	149 (74.5)	437 (61.9)	449 (62.1)	
Female	213 (41.5)	223 (42.6)	56 (29.0)	51 (25.5)	269 (38.1)	274 (37.9)	
Smoking status							< 0.0001
Current	228 (44.4)	142 (27.2)	88 (45.6)	77 (38.5)	316 (44.8)	219 (30.3)	
Former	59 (11.5)	79 (15.1)	20 (10.4)	24 (12.0)	79 (11.2)	103 (14.3)	
Non	226 (44.1)	302 (57.7)	85 (44.0)	99 (49.5)	311 (44.1)	401 (55.5)	
Drinking status							< 0.0001
Current	275 (53.6)	83 (15.9)	98 (50. 8)	57 (28.5)	373 (52.8)	140 (19.4)	
Former	14 (2.7)	25 (4.8)	12 (6.2)	10 (5.0)	26 (3.7)	35 (4.8)	
Non	224 (43.7)	415 (79.4)	83 (43.0)	133 (66.5)	307 (43.5)	548 (75.8)	
Family history of cancer							0.018
Yes	71 (13.8)	57 (10.9)	20 (10.4)	8 (4.0)	91 (12.9)	65 (9.0)	
No	442 (86.2)	466 (89.1)	173 (89.6)	192 (96.0)	615 (87.1)	658 (91.0)	
BMI (kg/m^2)							0.002
≤ 23.9	297 (57.9)	346 (66.2)	92 (47. 7)	118 (59.0)	389 (55.1)	464 (64.2)	
24.0-27.9	169 (32.9)	139 (26.6)	77 (39.9)	66 (33.0)	246 (34.8)	205 (28.4)	
≥ 28.0	47 (9.2)	38 (7.3)	24 (12.4)	16 (8.0)	71 (10.1)	54 (7.5)	
Menstrual history							0.881
Premenopause	43 (20.2)	41 (18.4)	10 (17.9)	11 (21.6)	53 (19.7)	52 (19.0)	
Menopause	170 (79.8)	182 (81.6)	46 (82.1)	40 (78.4)	216 (80.3)	222 (81.0)	

[1]*P* value for two-sided χ^2 test.

Table 2 Comparison of epidemiological factors for colorectal cancer

Variables	Adjusted OR (95% CI)[1]		
	Test group	Validation group	Merged group
Smoking status			
Non	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)
Former	0.97 (0.52-1.80)	0.66 (0.30-1.46)	0.78 (0.49-1.23)
Current	1.41 (0.82-2.43)	0.79 (0.45-1.38)	1.01 (0.69-1.48)
Trend test *P* value	< 0.00001	0.187	< 0.00001
Drinking status			
Non	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)
Former	2.02 (0.93-4.41)	2.84 (1.03-7.81)	2.30 (1.27-4.17)
Current	14.69 (9.41-22.94)	3.82 (2.23-6.56)	8.61 (6.15-12.05)
Trend test *P* value	< 0.00001	0.023	< 0.00001
Family history of cancer			
Negative	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)
Positive	1.43 (0.94-2.18)	3.21 (1.31-7.84)	1.62 (1.12-3.34)
BMI (kg/m^2)			
≤ 23.9	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)
≥ 24.0	1.34 (1.00-1.78)	1.60 (1.05-2.44)	1.39 (1.10-1.75)

[1]Adjusted for age, sex, smoking status, alcohol drinking, family history of cancer, BMI.

adjusted in multivariate logistic regression model for controlling any residual effect of possible confounding on the main effect of studied factors.

Risk factors for sporadic colorectal cancer

Logistic regression analysis showed that compared with the non alcohol drinkers, current and former alcohol drinkers had a significantly increased risk of developing sporadic CRC (adjusted OR = 8.61 and 95% CI = 6.15-12.05; adjusted OR = 2.30 and 95% CI = 1.27-4.17) (Table 2). Similarly, the increased risk of developing sporadic CRC was significantly greater in those with a positive family history of cancer (adjusted OR = 1.62, 95% CI = 1.12-3.34) and in those with their BMI ≥ 24.0 kg/m^2 (adjusted OR = 1.39, 95% CI = 1.10-1.75). There was a significant trend to develop CRC (P_{trend} < 0.00001) due to alcohol drinking. However, smoking status was not positively correlated with the risk of developing sporadic CRC.

Stratification analysis of colon and rectal cancer

We further performed a stratification analysis of the association between selected variables and risk of developing colon and rectal cancer in subgroups (Table 3). The risk of developing both colon and rectal cancers was increased in current alcohol drinkers (adjusted OR = 7.60 and 95% CI = 5.13-11.25; adjusted OR = 7.52 and 95% CI = 5.13-11.01) and in those with their BMI ≥ 24.0 kg/m^2 (adjusted OR = 1.38 and 95% CI = 1.04-1.83; adjusted OR = 1.35 and 95% CI = 1.02-1.79). The risk of developing colon cancer, but not rectal cancer, was found in former alcohol drinkers (adjusted OR = 2.51 and 95% CI = 1.24-5.07) and in those with a positive family history of cancer (adjusted OR = 1.82 and 95% CI = 1.17-2.82). However, smoking status was not significantly associated with the risk of developing CRC.

DISCUSSION

In our study, smoking status was not positively associated with the risk of developing sporadic CRC. Compared with the non alcohol drinkers, current and former alcohol drinkers had an increased risk of developing sporadic

Table 3 Stratification analysis of colon and rectal cancer

Variables	Adjusted OR (95% CI)[1]					
	Test group		Validation group		Merged group	
	Colon cancer (*n* = 253)	Rectal cancer (*n* = 260)	Colon cancer (*n* = 95)	Rectal cancer (*n* = 98)	Colon cancer (*n* = 348)	Rectal cancer (*n* = 358)
Smoking status						
Non	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)
Former	0.71 (0.34-1.49)	1.26 (0.59-2.69)	1.09 (0.44-2.72)	0.41 (0.14-1.18)	0.83 (0.48-1.43)	0.79 (0.45-1.38)
Current	1.34 (0.72-2.51)	1.87 (0.96-3.65)	1.03 (0.53-2.03)	0.78 (0.40-1.51)	1.22 (0.78-1.91)	1.17 (0.75-1.83)
Drinking status						
Non	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)
Former	2.28 (0.90-5.76)	1.32 (0.48-3.64)	2.51 (0.78-8.06)	3.10 (0.88-10.92)	2.51 (1.24-5.07)	1.71 (0.80-3.65)
Current	13.24 (7.78-22.51)	11.91 (7.14-19.87)	3.46 (1.83-6.53)	3.61 (1.94-6.74)	7.60 (5.13-11.25)	7.52 (5.13-11.01)
Family history of cancer						
Negative	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)
Positive	1.67 (1.01-2.75)	1.26 (0.76-2.09)	3.26 (1.12-9.45)	3.52 (1.26-9.85)	1.82 (1.17-2.82)	1.51 (0.97-2.35)
BMI (kg/m^2)						
≤ 23.9	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)
≥ 24.0	1.48 (1.05-2.09)	1.22 (0.86-1.73)	1.18 (0.70-1.99)	2.00 (1.18-3.36)	1.38 (1.04-1.83)	1.35 (1.02-1.79)

[1]Adjusted for age, sex, smoking status, alcohol drinking, family history of cancer, BMI.

CRC. The increased risk of developing sporadic CRC was found in those with their BMI ≥ 24.0 kg/m^2 and in those with a positive family history of cancer. The stratification analysis showed that the risk of developing both colon and rectal cancers was increased in current alcohol drinkers and in those with their BMI ≥ 24.0 kg/m^2. The increased risk of developing colon cancer but not rectal cancer was found in former alcohol drinkers and in those with a positive family history of cancer.

It has been reported that smoking is related with the risk of developing invasive colorectal cancer and current smokers have a significantly increased risk of developing rectal cancer but not colon cancer compared with non smokers[12]. In a population-based case-control study recruiting 540 cases of colorectal cancer and 614 controls in Germany, current smokers had a significantly increased risk of developing colorectal cancer compared with non smokers[13]. Another report including 852 patients demonstrated that tobacco smoking is a risk factor for early colorectal cancer[14]. Inconsistent with the above reports, in our study, smoking was not associated with the risk of developing colorectal cancer. However, our results are consistent with the reported findings[7]. The different results might be due to the different time of smoking between mainland Chinese and other populations. The main increase in cigarette consumption in various Chinese communities occurred a few decades later than that of the Western countries[15]. Similarly, the prevalence of cigarette smoking reached its peak in Hong Kong about 20 years earlier than in mainland China[15]. Giovannucci *et al*[16,17] demonstrated that smoking is related to the risk of developing colorectal cancer only after allowing for an induction period of at least 35 years. It has been shown that long-term smoking is associated with the elevated risk of developing colorectal cancer[13,18].

Our results suggest that alcohol drinking was an independent risk factor for sporadic CRC, which is consistent with the findings in Japan, American, French, and Hong Kong[3,19-21]. However, the OR value in our study was much higher than the reported data, which may be due to the selection bias in our study. In our study, the cases were recruited from 6 hospitals, whereas the controls were enrolled from communities in Guangzhou City. Thus, the percentage of current alcohol drinkers in the controls was relatively low (Table 1).

Our study found that there was an independent association between BMI and sporadic CRC risk in Chinese. Insulin action decreases with increasing obesity[22]. Insulin resistance develops as a metabolic adaptation to increased levels of circulating non-esterified fatty acids released from adipose tissues. Because non-esterified fatty acids force the liver, muscles, and other tissues to store and oxidize fats for energy, the pancreas would secrete more insulin to prevent elevated concentrations of glucose in blood[23]. Increased blood insulin levels decrease insulin-like growth factor binding protein 1 levels, thus increasing free insulin-like growth factor 1 (IGF-1) levels[24]. It has been shown that insulin resistance is related with hyperinsulinaemia, IGF-1 and colorectal cancer[25-27]. In this study, the increased risk of developing colon cancer but not rectal cancer was associated with a positive family history of cancer, indicating that genetic susceptibility may play a more important role in the pathogenesis of colon cancer than in that of rectal cancer.

However, our study had several limitations, such as a small sample size, hospital-based case control, Chinese subjects, and lack of food intake information, which might result in improper findings.

In conclusion, in our study, we found that alcohol drinking and greater BMI (≥ 24.0 kg/m^2) are the independent risk factors for colon and rectal cancer in southern Chinese. The risk of developing colon cancer but not rectal cancer increases in former alcohol drinkers and in those with a positive family history of cancer. Because of the uncontrolled bias in selection of participants and retrospective design, our findings need to be further evaluated in well-designed larger epidemiological studies with different ethnic populations.

COMMENTS

Background

The incidence of colorectal cancer in China is growing. More attention should be paid to the prevention and control of colorectal cancer. However, the epidemiological factors for colorectal cancer are controversial.

Research frontiers

Although the association between the epidemiological factors and sporadic colorectal cancer has been studied, the relation between smoking, alcohol drinking, family history of cancer, body mass index (BMI) and sporadic colorectal cancer still remains uncertain. It is important to investigate the role of these factors in the development of sporadic colorectal cancer.

Innovations and breakthroughs

In this study, the authors found that current alcohol drinking and greater BMI ($\geqslant$ 24.0 kg/m^2) are the independent risk factors for colon and rectal cancer, while former alcohol drinking and positive family history of cancer are the independent risk factors for colon cancer in southern Chinese.

Peer review

In the case-control study, smoking, alcohol consumption, BMI and family history of cancer were evaluated in patients with sporadic colorectal cancer. The study showed that alcohol drinking, higher BMI ($\geqslant$ 24.0 kg/m^2) and positive family history of cancer were the independent risk factors for sporadic colorectal cancer in a southern Chinese. Its findings may contribute to the prevention and control of sporadic colorectal cancer.

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Barriers to colorectal cancer screening: A case-control study

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Abstract

AIM: To investigate barriers to colorectal cancer (CRC) screening in a community population.

METHODS: We conducted a community-based case-control study in an urban Chinese population by questionnaire. Cases were selected from those completing both a fecal occult blood test (FOBT) case and colonoscopy in a CRC screening program in 2004. Control groups were matched by gender, age group and community. Control 1 included those having a positive FOBT but refusing a colonoscopy. Control 2 included those who refused both an FOBT and colonoscopy.

RESULTS: The impact of occupation on willingness to attend a colorectal screening program differed by gender. *P* for heterogeneity was 0.009 for case *vs* control group 1, 0.01 for case versus control group 2, and 0.80 for control group 1 *vs* 2. Poor awareness of CRC and its screening program, characteristics of screening tests, and lack of time affected the screening rate. Financial support, fear of pain and bowel preparation were barriers to a colonoscopy as a screening test. Eighty-two percent of control group 1 and 87.1% of control group 2 were willing attend if the colonoscopy was free, but only 56.3% and 53.1%, respectively, if it was self-paid. Multivariate odds ratios for case *vs* control group 1 were 0.10 among those unwilling to attend a free colonoscopy and 0.50 among those unwilling to attend a self-paid colonoscopy.

CONCLUSION: Raising the public awareness of CRC and its screening, integrating CRC screening into the health care system, and using a painless colonoscopy would increase its screening rate.

Key words: Colorectal cancer screening; Barrier; Community-based case-control study; Colonoscopy; Fecal occult blood test

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INTRODUCTION

Colorectal cancer is the second to fifth cause of cancer death in urban populations among different cities in China. The incidence and mortality of colorectal cancer is increasing rapidly in developing countries[1,2]. Recently, strong evidence from several randomized intervention studies indicated that colorectal cancer screening is effective in reducing its mortality[3-7]. However, the reported compliance of colorectal cancer screening in the general population varies widely and is generally low. The reported participation rate for fecal occult blood tests (FOBT) ranges from 12% to 95% in community-based programs and from 12% to 27% for a flexible sigmoidoscopy[8-14].

In 2004, we carried out a free community-based colorectal cancer screening program in Hangzhou city in China, where the target population (38 337

people) was 40-74 years old. 14269 people accepted an immunochemical FOBT for colorectal cancer screening (participation rate was 37.2%) and of the 509 people who were positive, only 94 people accepted a free colonoscopy, a participation rate of 18.5% (94/509). Thus, 415 people refused the free colonoscopy[15]. Though poor knowledge about colorectal cancer, social factors and the test provider might have influenced compliance of colorectal cancer screening[16-21], it is not clear why people are unwilling to attend the colorectal cancer screening program in China. To better understand why people are unwilling to attend the colorectal cancer screening program in China, we conducted this study to explore the barriers to conducting a colorectal cancer screening.

MATERIALS AND METHODS

Study design and population

We conducted a population-based case-control study of barriers to colorectal cancer screening in Hangzhou city in China. Cases were selected from those who underwent both an FOBT and a colonoscopy in a previous colorectal cancer screening program, resulting in a total of 94 subjects. All permanent residents in the Changqing and Chaoming communities in the Xiachen district of Hangzhou city, aged from 40 to 74 years old, were invited to attend a free colorectal cancer screening program in 2004[15]. A two-step screening method was applied in this screening program. Immunochemical FOBT and a questionnaire of high-risk factors were used in the first step. If the FOBT was positive or the questionnaire reported high-risk factors [including other cancer or polypi history; or a family history of colorectal cancer among the first relatives; or at least two of the following histories: chronic coprostasis, chronic diarrhea, mucous bloody feces, stressful life events (such as divorce and deaths among the first relatives); and chronic appendicitis] then a colonoscopy without any auxiliary medicine was suggested as the second step. Only 11 subjects at the first step were identified as high-risk population by the questionnaire, so they were excluded from this study. Every qualified subject from the first step or the second step of screening was invited three times (orally, by letter, and by telephone invitation). If subjects refused all three invitations, we defined them as refusers that could be included as a control.

Two sets of control groups were designed to match the case group by gender, age group, and community location. Age groups were recorded into groups with an initial age of 40 years old by intervals of 5 years. Control group 1 included those had a positive FOBT test but refused a colonoscopy. Control group 2 included those who refused both an FOBT test and colonoscopy. We were able to accurately select cases and controls from the defined communities because there is a complete registration of all permanent residents in every district in Hangzhou city.

All subjects were asked to complete a questionnaire by interview in-person by fixed interviewers who were well-trained in advance. All data were recorded, numerically transformed into computer files at least three times, and their reliability confirmed for analysis. Data collection was performed in 2006-2007.

Table 1 Characteristics of subjects between case and control groups[1]

Characteristic	Case	Control 1	Control 2	*P*-value
Age (mean ± SD, yr)	53.3 ± 9.0	54.3 ± 8.3	52.7 ± 9.7	0.23
Gender				
Male	47	70	98	
Female	39	94	115	0.19
Marital status				
Ever	83	163	208	
Never	3	1	5	0.25
Occupation				
Worker	19	65	69	
Official/administrator	3	3	9	
Technician/professional	7	8	6	
Businessman	8	11	11	
Other	49	77	18	0.08
Education				
College & above	12	18	23	
High school	17	29	34	
Middle school	45	94	132	
Elementary school or none	12	23	24	0.65
Personal income[2]				
≤ ¥10000	53	92	134	
¥10001-20000	21	45	51	
> ¥20000	9	16	21	0.53

[1]Definition: case, those completing both a fecal occult blood test (FOBT) and colonoscopy; control 1, those having a positive FOBT but refusing a colonoscopy, and control 2, those who refused both an FOBT and colonoscopy in a CRC screening program in 2004. [2]Estimated by yearly household income in Chinese Dollar (Yuan) divided by total number of family members.

Statistical analysis

The STATA 8.0 program was used for data analysis. One-way ANOVA was used to compare means between case and control groups. Chi-square (χ^2) was used for frequency data. If data were not suitable for χ^2 analyses, a Mann-Whitney *U* test was used to compare the ratio between two groups and a Kruskal-Wallis test was used in multiple groups' ratio comparison. Pearson *P* values were estimated for basic characteristics and logistic regression (because we did not match exactly in 1:2 or 1:3, we did not use conditional logistic regression) was used to estimate the odds ratios and 95% confidence intervals for each variable. A likelihood ratio test was used to test heterogeneity..

RESULTS

In the case group, 86 subjects (8 subjects refused participation) were finally included in our analysis. In the control groups, 164 and 213 subjects were included in control 1 and 2, respectively. Overall, there were no significant differences in age, gender, education level, household income, and occupation between the case and control groups based on Pearson *P* values (Table 1). However, cases had a higher percentage of white-collar employees than control groups, including official/

Table 2 Differences by gender in association of occupation and willingness to attend a colorectal screening program

	OR1 (95% CI)[1]			OR2 (95% CI)[1]			OR3 (95% CI)[1]		
	Together[2]	Male	Female	Together[2]	Male	Female	Together[2]	Male	Female
Occupation									
Worker	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Official/administrator	3.42	4.13	-	1.21	3.40	0.50	2.83	1.22	-
	(0.64-18.36)	(0.55-31.26)		(0.30-4.92)	(0.49-23.65)	(0.05-4.67)	(0.73-10.90)	(0.25-5.87)	
Technician/professional	2.99	15.5	0.81	4.24	6.80	5.00	0.71	2.28	0.16
	(0.96-9.32)	(2.34-102.85)	(0.15-4.51)	(1.27-14.11)	(1.44-32.20)	(0.42-59.66)	(0.23-2.15)	(0.41-12.61)	(0.02-1.42)
Businessman	2.49	12.4	0.61	2.64	8.16	0.83	0.94	1.52	0.73
	(0.88-7.07)	(2.32-66.35)	(0.11-3.22)	(0.93-7.49)	(1.80-37.06)	(0.15-4.64)	(0.38-2.32)	(0.34-6.89)	(0.23-2.32)
Other	2.18	5.8	1.06	1.51	3.94	0.74	1.44	1.47	1.44
	(1.17-4.06)	(1.99-16.94)	(0.47-2.38)	(0.82-2.77)	(1.39-11.21)	(0.33-1.63)	(0.93-2.25)	(0.76-2.85)	(0.79-2.62)
Gender									
Male	1.00			1.00			1.00		
Female	0.62			0.71			0.87		
	(0.37-1.04)			(0.43-1.17)			(0.58-1.32)		
P for heterogeneity	0.009			0.01			0.80		

[1]OR1: Odds ratio for case *versus* control 1; OR2: Odds ratio for case *versus* control 2; OR3: Odds ratio for comparison between two control groups; 95% CI means 95% confidence interval. [2]OR's in all means only occupation and gender factors were single in the models, or called a single OR.

administration, technician/professional, businessman, and other occupations. Where the difference between case and controls groups differed by gender, white-collar males tended to be more willing to participate in the colorectal cancer screening program than blue-collar men, but females were not (Table 2).

More people in the case group than in the two control groups thought that: 1: colorectal cancer is a common cancer [The adjusted odds ratio for the answer of "I don't know colorectal cancer is a common cancer" was 0.22 (95% CI: 0.09-0.56) for case versus control 1 and 0.24 (0.10-0.54) for case versus control 2 compared to the reference group of answering "certainly know"]; 2: screening will help improve the consciousness of health; 3: screening can find an early stage of colorectal cancer; and 4: it is necessary to conduct the colorectal cancer screening in the community (Table 3). However, there was no difference in their knowledge of the existence of this screening program between cases and controls. A Kruskal-Wallis test was used to analyze one-direction data. Based on the results from the Kruskal-Wallis test, people in the case group had more knowledge of colorectal cancer and its screening, and were defined as "good". Control group 1 was defined as "fair" and control group 2 as "poor". Overall, there was no significant difference in knowledge of colorectal cancer and its screening between the two control groups.

There were no significant differences in the inclination to attend colorectal cancer screening between the case and control groups if using self-paid FOBT as a screening test (Table 4). However, if using colonoscopy as a screening test, the percentage willing to participate in the screening program was higher among cases than controls under the same payment method. The rate of willingness to attend a colonoscopy was higher if the colonoscopy was free than if it was self-paid. The rate was 82.0% among control group 1 and 87.1% among control group 2 if the colonoscopy was free, whereas it declined to 56.3% and 53.1%, respectively, if the colonoscopy was self-paid.

Finally, we investigated why people refused a colonoscopy. There were no significant differences in reasons for refusing a colonoscopy between two control groups with Mann-Whitney *U* test ($Z = 2.51$, $P = 0.11$), so we combined the two control groups together. Approximately 46.4% of people who declined to participate in the screening program reported that they lacked time. Approximately 20.8% of people were unwilling or unable to pay for a colonoscopy and future therapy. Fear of bowel preparation (5.0%) and pain (11.1%) were also major reasons. Only 7.5% of people reported that the screening test is included in their routine health examination and 4.6% refused a colonoscopy because they thought it was of no significance. Approximately 4.3% of people reported no interest.

DISCUSSION

Our study investigated the barriers to conducting a colorectal cancer screening program in an urban population, aged between 40 and 74 years, in China. We found that white-collar men tended to be more willing to participate in the screening program than blue-collar men. Poor awareness of colorectal cancer and its screening program, the characteristics of the screening tests, lack of time, and financial issues were important factors that affected the attendance rate of colorectal cancer screening programs. Education and household income did not affect the screening rate in this senior urban population.

Our results are reliable and generally applicable because this is a community-based case-control study with two sets of control groups and high quality assurance and control, which nested in a free population-based colorectal cancer screening program conducted in the same area. The participation rate, 91.5%, was high. All subjects completed a questionnaire when interviewed in person by well-trained interviewers. All data were recoded, numerically transformed into computer files at least three

Table 3 Status of knowledge about colorectal cancer and its screening between case and control groups

Item[1]	Case	Control 1	Control 2	OR1 (95% CI)[2]		OR2 (95% CI)[2]		OR3 (95% CI)[2]	
				Single	Multiple	Single	Multiple	Single	Multiple
Question 1									
Yes, certain	57	84	68	1.0	1.0	1.0	1.0	1.0	1.0
Heard of it	14	30	77	0.69 (0.34-1.41)	0.50 (0.21-1.17)	0.22 (0.11-0.42)	0.15 (0.07-0.33)	3.17 (1.87-5.38)	2.90 (1.62-5.21)
I don't know	15	50	68	0.44 (0.23-0.86)	0.22 (0.09-0.56)	0.45 (0.24-0.84)	0.24 (0.10-0.54)	1.68 (1.03-2.73)	1.49 (0.80-2.80)
Question 2									
Yes, a lot	65	88	78	1.0	1.0	1.0	1.0	1.0	1.0
Yes, some	20	71	131	0.38 (0.21-0.68)	0.25 (0.12-0.54)	0.18 (0.10-0.32)	0.13 (0.06-0.27)	2.06 (1.35-3.13)	2.06 (1.21-3.50)
No	0	5	3	-	-	-	-	0.67 (0.15-2.89)	0.52 (0.11-2.56)
Question 3									
Yes, certain	41	55	54	1.0	1.0	1.0	1.0	1.0	1.0
Yes, possible	45	94	151	0.64 (0.37-1.10)	0.71 (0.36-1.39)	0.40 (0.24-0.67)	0.38 (0.20-0.72)	1.61 (1.02-2.53)	1.77 (1.04-3.02)
No, impossible	0	15	7	-	-	-	-	0.47 (0.18-1.23)	0.43 (0.14-1.38)
Question 4									
Yes	79	138	153	1.0	1.0	1.0	1.0	1.0	1.0
No	0	7	6	-	-	-	-	2.50 (1.41-4.43)	2.11 (1.12-3.99)
Don't care	7	19	53	0.64 (0.26-1.60)	0.58 (0.20-1.70)	0.26 (0.11-0.59)	0.25 (0.10-0.62)	0.77 (0.25-2.34)	0.68 (0.21-2.23)
Question 5									
Community	81	160	197	1.0	1.0	1.0	1.0	1.0	1.0
News media	4	3	9	2.65 (0.58-12.13)	2.52 (0.44-14.49)	1.07 (0.32-3.56)	1.22 (0.32-4.60)	2.48 (0.66-9.33)	2.79 (0.67-11.58)
Both	1	1	5	1.99 (0.12-32.19)	2.43 (0.08-75.03)	0.40 (0.05-3.38)	0.40 (0.03-4.54)	4.97 (0.59-41.70)	4.11 (0.47-36.29)

[1]Question 1: Do you think colorectal cancer is a common cancer? Question 2: Do you think this screening will help you improve your awareness of health? Question 3: Do you think this screening can find an early stage of colorectal cancer? Question 4: Do you think it is necessary to conduct the colorectal cancer screening in your community? Question 5: How do you know this screening program? Community means community screening center; [2]All single OR's means without adjustment for any other factors in the model, and all multiple OR's means with adjustment for the following factors including age (0 = < 45, 1 = 45-49, 2 = 50-54, 3 = 55-59, 4 = 60-64, 5 = 65-69, and 6 = ⩾ 70), gender (0 = male and 1 = female), occupation (0 = worker, 1 = official/administrator, 2 = technician/professional, 3 = businessman, and 4 = others), interaction term between gender and occupation, and annual personal income (0 = < ¥10 000, 1 = ¥10,000-< 20 000, and 2 = ⩾ ¥20 000); all variables were treated as dummy variables in the logistic regression model.

Table 4 Attendance inclination of colorectal cancer screening with different payment methods between case and control groups

Inclination to attend	Case	Control 1	Control 2	OR1 (95% CI)		OR2 (95% CI)		OR3 (95% CI)	
				Single	Multiple	Single	Multiple	Single	Multiple
If FOBT self-paid									
Yes	45	84	99	1.0	1.0	1.0	1.0	1.0	1.0
No	37	73	107	0.95 (0.55-1.62)	0.98 (0.53-1.79)	0.76 (0.46-1.27)	0.66 (0.37-1.19)	1.24 (0.82-1.89)	1.37 (0.86-2.19)
Refused[1]	4	7	7						
If free colonoscopy									
Yes	81	132	162	1.0	1.0	1.0	1.0	1.0	1.0
No	2	29	24	0.11 (0.03-0.48)	0.10 (0.02-0.45)	0.17 (0.04-0.72)	0.14 (0.03-0.65)	0.67 (0.37-1.21)	0.70 (0.36-1.35)
Refused[1]	3	3	27						
If colonoscopy self-paid									
Yes	57	85	93	1.0	1.0	1.0	1.0	1.0	1.0
No	23	66	82	0.52 (0.29-0.93)	0.50 (0.26-0.97)	0.46 (0.26-0.81)	0.43 (0.23-0.80)	1.14 (0.73-1.76)	1.12 (0.68-1.83)
Refused[1]	6	13	38						

[1]Refers to those who refused to answer this question; results in this row not concluded in statistical analysis.

times, and their reliability was confirmed for analysis. Data analyses were performed blindly by two analysts. However, the sample size is limited by the low colorectal cancer screening rate in the general population.

Five randomized intervention studies demonstrated that screening could reduce colorectal cancer mortality[3-7]. However, screening rates in the general population are low. In the USA, only 32% of adults over age 50 years has had a FOBT in the past two years and only 34% say they have ever had either a sigmoidoscopy or colonoscopy for some reason[22]. In our pilot study, the screening rate at the primary stage reached 50% and at secondary stage (colonoscopy) it was nearly 20%. Overall, completion in all tests used in colorectal cancer screening are not satisfactory[23]. Why people refuse colorectal cancer screening is of great importance to improving the efficacy of colorectal cancer screening.

In our study, we found that education and household income did not affect the screening rate. Some social characteristics such as low education are risk factors in ovarian cancer and breast cancer screening[24,25]. Occupation tended to be a risk factor that affected the screening rate more for men than for women. White-collar men might have higher awareness of colorectal cancer and its screening and less of a financial issue than blue-collar men. Our results indicated that poor

knowledge of colorectal cancer screening was a major barrier to improving the screening rate, which is supported by reports that poor knowledge of colorectal cancer screening is a prognostic factor of screening rates in Chinese communities in Singapore[16,17]. Public education about colorectal cancer, risk factors, and potential benefits from colorectal cancer screening should be performed. Access to screening programs was similar in the three groups. The screening program center played a major role in screening invitations.

In this community-based study, the attendance rate of colorectal cancer screening was affected by multiple factors including characteristics of the screening test, financial issues, fear of pain and bowel preparation, lack of time, and poor awareness of risk factors, screening guidelines and screening importance. If an FOBT was used as a screening test, the screening rates were similar between case and control groups whether this screening was free or not. Attendance rate declined if a colonoscopy was used as a screening test. The attendance rate declined more if the colonoscopy was self-paid. FOBT is a cheap, painless, and convenient test. The screening rate would increase in China if a painless colonoscopy were used in screening programs. Integration of colorectal cancer screening into primary care practice or the Medicare system could also facilitate colorectal cancer screening, which is suggested in both our study and other studies[18,19].

In the two step colorectal cancer screening program, neither the first nor the second step is indispensable. A FOBT is more adaptable than a colonoscopy and a flexible sigmoidoscopy in colorectal cancer screening[20], mainly because any endoscopies need bowel preparation and have some degree of discomfort. The attendance rate for colonoscopies is relatively low compared to that of FOBTs in China. The attendance rate of colonoscopies is high in the USA[23]. This might be due to the recent U.S. national Medicare coverage and wide application of a painless colonoscopy in USA.

In summary, by improving public health education of cancer, integrating colorectal cancer screening into the Medicare and or primary care systems, and applying anesthesia in colonoscopies, there would be an increase in the colorectal cancer screening rate, leading to a possible decrease in the mortality rate of colorectal cancer in the long-term.

COMMENTS

Background

Recently, strong evidence from several randomized intervention studies indicated that colorectal cancer screening is effective in reducing its mortality. However, the reported compliance of colorectal cancer screening in the general population, including China, varies widely and is generally low. It is not clear why people are unwilling to attend the colorectal cancer screening program in China.

Innovations and breakthroughs

The impact of occupation on willingness to attend a colorectal screening program differs by gender. Poor awareness of colorectal cancer (CRC) and its screening program, characteristics of screening tests, and lack of time were important factors affecting the screening rate in the community. Financial support and fear of pain and bowel preparation were important barriers to a colonoscopy as a screening test. Raising public awareness of CRC and its screening, integrating CRC screening into health care system, and applying a painless colonoscopy would increase the screening rate.

Terminology

Fecal occult blood is a term for blood present in the faeces that is not visibly apparent. In medicine, a fecal occult blood (FOBT) test is a check for hidden (occult) blood in the stool. FOBT testing can provide clues as to subtle blood loss in the gastrointestinal tract, anywhere from the mouth to the colon. Positive tests warrant further investigation for peptic ulcers or a malignancy (such as colorectal cancer or gastric cancer).

Peer review

This is an important paper looking at barriers to CRC screening. It is interesting to note that financial considerations in China seem to be much more important than in the US. This might be due to the fact that many Chinese patients have to pay for screening procedures out of their pockets. The results imply that improving health education and expanding the coverage of Medicare and primary care systems could increase the colorectal cancer screening rate, and could decrease the mortality of colorectal cancer in the long-term.

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Rapid detection of intestinal pathogens in fecal samples by an improved reverse dot blot method

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Abstract

AIM: To develop a new, rapid and accurate reverse dot blot (RDB) method for the detection of intestinal pathogens in fecal samples.

METHODS: The 12 intestinal pathogens tested were *Salmonella* spp., *Brucella* spp., *Escherichia coli* O157:H7, *Clostridium botulinum*, *Bacillus cereus*, *Clostridium perfringens*, *Vibrio parahaemolyticus*, *Shigella* spp., *Yersinia enterocolitica*, *Vibrio cholerae*, *Listeria monocytogenes* and *Staphylococcus aureus*. The two universal primers were designed to amplify two variable regions of bacterial 16S and 23S rDNA genes from all of the 12 bacterial species tested. Five hundred and forty fecal samples from the diarrhea patients were detected using the improved RDB assay.

RESULTS: The methods could identify the 12 intestinal pathogens specifically, and the detection limit was as low as 103 CFUs. The consistent detection rate of the improved RDB assay compared with the traditional culture method was up to 88.75%.

CONCLUSION: The hybridization results indicated that the improved RDB assay developed was a reliable method for the detection of intestinal pathogen in fecal samples.

Key words: Immunoblotting; Intestinal pathogens; Feces

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INTRODUCTION

Foodborne infections are an important public health concern worldwide. The World Health Organization and the US Centers for Disease Control and Prevention (CDC)[1,2] report every year a large number of people affected by diseases caused by intestinal pathogens that have contaminated food. The main clinical manifestations of infection with intestinal pathogens are nausea, vomiting and diarrhea. The clinical syndromes caused by the different intestinal pathogens are usually not distinguishable[3,4]. Therefore, identification of intestinal pathogens is heavily dependent on help from clinical laboratories. Most intestinal pathogens are difficult to incubate under the same conditions[5]. Furthermore, the current assays for identifying pathogens are performed mainly using cultivation of cells. Although the cultivation has been a standard method of pathogenic identification, the whole procedure takes around 5 d, or even longer, to obtain final results. The time-consuming procedure not only makes the methods difficult for high-throughput use, but also involves specialized techniques and expertise[6]. As a result, the patients would probably lose the optimal chance of therapy, and centers for disease control would not be able to take effective measures rapidly. Consequently, considerable effort should be devoted to establish rapid, sensitive and specific assays for identifying intestinal pathogens.

At present, many methods have been developed to detect intestinal pathogens, such as mass spectrometric analysis[7], fluorescence polarization[8,9], real-time fluorescence quantitative PCR[10,11], microarray[12,13], and sequencing. Most of these techniques are accurate, but time-consuming, labor-intensive, and hard to adapt to

high-throughput screening. They are only amenable to analysis by those who are well-trained and well-equipped, which is not suitable for small hospitals. Nevertheless, the reverse dot blot (RDB) method can be used to detect many pathogens simultaneously. As bacterial 16S and 23S rDNA genes, bacterial live fossils, have great significance in taxonomy[14]. These two genes have been less changeable than others in the course of evolution. We combined flow-through hybridization technology with RDB assay to develop a rapid RDB method that can simultaneously detect 10 intestinal pathogens according to the bacterial 16S and 23S rDNA genes. Compared with the conventional passive hybridization process that required hours or even overnight hybridization, the flow-through hybridization takes only several minutes to complete, by directing the flow of the target molecules toward the immobilized probes.

MATERIALS AND METHODS

Bacterial strains and clinical samples

Bacterial reference strains were obtained from the National Institute for the Control of Pharmaceutical and Biological Products of China (Table 1), and chosen from a wide range of genera or species. All the strains selected were cultured for 24-36 h according to conventional methods[15,16]. All fecal samples were collected from 540 patients who had diarrhea from May 2006 to July 2007, at the Central Hospital in Huzhou, China. The clinical samples were isolated and identified by conventional methods and, except for the coagulase-negative *Staphylococcus aureus*, by the appropriate API test system.

Design of primers and pathogen-specific oligonucleotide probes[17]

The primers were designed using Primer 5.0 software on conservative regions based on the *Escherichia coli* (*E. coli*) 16S rDNA and 23S rDNA (GenBank accession number U00096). All oligonucleotide probes were designed from variable regions between two pairs of primers of each pathogen available in the GenBank database (GenBank/EMBL/DDBJ). Multiple-sequence alignments were carried out by using the ClustalW program. By comparison of the sequences of the 16S and 23S rDNA regions of the target species, regions with interspecies variations could be identified and were used to develop species-specific probes.

The reverse primer was labeled with biotin at the 5' end, and the hybridization probes were labeled with amino group at the 5' end. In order to judge the validity of the hybridization process, we designed a color control probe for the hybridization control, which was labeled with a biotin group at the 5' end and an amino group at the 3' end. The color control probe can bind only with the chromogen but not with the targeting molecule. All oligonucleotide primers (Table 2) and probes (Table 3) were synthesized commercially at Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China).

Table 1 Standard strains used in the present study

Genus or species	Standard strain ATCC accession no.
S. aureus	26001, 26111, 26113
V. cholerae	16025, 16026, 16028
Shigella spp.	51081, 51207, 51335
E. coli O157:H7	44752, 43889, 43859
V. parahaemolyticus	20502, 20506, 20507
Salmonella spp.	50001, 50004, 50013
Y. enterocolitica	52207, 52211, 52215
L. monocytogenes	54003, 54005, 54006
Brucella spp.	23456
C. botulinum	64201, 64203
B. cereus	63301, 6051, 63509
C. perfringens	64711, 13048

Table 2 Universal primers used in the present study

Primer name	Sequence (5'→3')	PCR product size (bp)
16SF	CGCTGGCGGCAGGCCTAACACATGC	500
16SR	Biotin-GCGGCTGCTGGCACGGAGT-TAGCC	
23SF	ACCGATAGTGAACCAGTACCGTGAG	640
23SR	Biotin-TTAAATGATGGCTGCTTCTA-AGCC	

DNA isolation and PCR amplification

Processing of the fecal samples and subsequent bacterial DNA extraction using the QIAamp DNA Stool Mini Kit (Qiagen) and the genomic DNA of bacterial reference strains was extracted using the QIAamp DNA Mini Kit (Qiagen). Five microliters of the DNA was amplified by PCR in 50 μL of 1 × PCR buffer that contained 200 μmol/L of each dNTP, 2 U *Taq* DNA polymerase (Takara), 0.06 μmol/L forward primers (23S-F and 16S-F) and 0.3 μmol/L reverse primers (16S-R and 16S-R). In order to prevent contamination, we replaced dTTP with dUTP and added 0.5 U uracil-DNA glycosylase (UDG) to the PCR system. The amplification was performed by using an Applied Biosystems 9600 thermal cycler (Perkin-Elmer) under the following conditions: incubation at 50℃ for 3 min, before an initial denaturation step at 94℃ for 5 min, followed by 35 cycles of 94℃ for 30 s, 55℃ for 45 s, and 72℃ for 45 s. A final extension was performed at 72℃ for 5 min.

Membrane preparation and subsequent immobilization of oligonucleotides

Biodyne C membranes (Pall Co.) were rinsed briefly with 0.1 mol/L HCl, and then treated for 15 min with freshly prepared 20% EDC (w/v) [*N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride; Sigma-Aldrich, commercial grade] in deionized water, rinsed with deionized water, and the amino-modified oligonucleotide probes dissolved in 0.5 mol/L sodium bicarbonate buffer (pH 8.4) were dotted at the given positions on the membrane (Figure 1). The amino group of the probe may bind with the carboxyl group of the membrane. The dots were rinsed with Tris-buffered saline/0.1%

Table 3 Oligonucleotide probes used in the present study

Probe	Sequence (5′→3′)	Target
1	GGGAGTAAAGTTAATACCTTTGCTCATTGA	*Salmonella* spp.
2	CACACTGGAACTGAGACACGGTCGAGACTCCTACG GGA	Bacterial universal probe
3	CGTACCATTTGCTACGGAATAACTCAGGGAAACTTGTG	*Brucella* spp.
4	TCACCCCATAAAAGAGGCTCCCACTGC	*E. coli* O157:H7
5	TATAAGAGAATCGCATGATTTTCTTATCCAAAGATTTAT	*C. botulinum*
6	TGCTAGTTGAATAAGCTGGCACCTTGACG	*B. cereus*
7	ATGGCATCATCATTCAACCATTGGAGCAATCCGCTATGAGATGGACCC	*C. perfringens*
8	GGTTTCAGGTTCTTTTTCACTCCCCTCGCCG	Common probe for Shigella and *Salmonella* spp.
9	AAACGAGTTATCTGAACCTTCGGGGAACGATAACGG	*V. parahaemolyticus*
10	GAAGGCCTTTTCGATAATGATACCGGCGCTCTGCTCTCCC	*Shigella* spp. and Enteroinvasive *E. coli*
11	GGTGTTGTGGTTAATAACCGCAGCAATTGA	*Shigella* spp.
12	CTTCAATAATGCCAGCAGCTCCAACCCCGAAATAGATA	*Salmonella typhi*
13	CATAAAGGTTAATAACCTTTGTGATTGACGT	*Y. enterocolitica*
14	GCGGCAGCGGGAAGTAGTTTACTACTTTGCCGG	*Yersinia* spp.
15	CAGCACAGAGGAACTTGTTCCTTGGGTGGCGAG	*V. cholerae*
16	TGTTGTTAGAGAAGAACAAGGATAAGAGTAACTGCT	*L. monocytogenes*
17	ACATATGTGTAAGTAACTGTGCACATCTTGACGGTA	*S. aureus*
P	TTTGGCTAACTCCGTGCCAGCAGCCGCG	Positive control
C	BIOTIN-CCGCTGTATCACAAGGGCTGGTACCTTT	Color control
N	TTTCCGCTGTATCACAAGGGCTGGTACC	Negtive control
B	0.5 mol/L sodium bicarbonate buffer	Blank control

Ⓟ	Ⓑ	Ⓝ	Ⓒ
①	②	③	④
⑤	⑥	⑦	⑧
⑨	⑩	⑪	⑫
⑬	⑭	⑮	⑯
Ⓒ	Ⓝ	⑰	Ⓟ

Figure 1 Layout of oligonucleotide probes. Their sequences are indicated in Table 3.

Tween-20. Any remaining active groups were quenched with 0.1 mol/L NaOH for 10 min. Finally, filters were rinsed with deionized water and air-dried for storage, or were used immediately for hybridization.

RDB and flow-through hybridization

The improved RDB method was used according to the principle of flow-through hybridization, which was performed on the KaiPu DNA hybriMax Rapid Hybridization Machine (Hong Kong DNA Ltd., Hong Kong, China). Its detailed steps were as follows: (1) denature the PCR products (or omitted); (2) prehybridize the membrane (or omitted); (3) hybridize the target PCR products with the specific probes at 42℃ for 15 min; (4) wash the unhybridized PCR products; (5) combine peroxidase (POD) with the biotin group on the PCR products or on the color control probe at 37℃ for 5 min; (6) wash the membrane to eliminate the uncombined POD; and (7) color with 3,3,5,5-tetramethylbenzidine (TMB) chromogen. We set positive and negative controls for all detections. The machine worked on the basis of the particular principle of flow-through hybridization; there was a negative pressure under the airproof hybridization membrane, which was produced by pumping. All of the hybridization solution, washing solution, POD solution, and coloring solution flowed through the membrane automatically. The improved RDB method actively directed the flow of the targeting molecules toward the immobilized probes within the membrane fibers. The complementary molecules were hybridized and formed duplex DNA; at the same time, any unbound molecules were removed by passing through the membrane. This speeded up the interaction between the complementary molecules, reduced the hybridization time from hours down to minutes, and provided results hundreds of times faster than by using traditional passive hybridization methods.

RESULTS

Dual PCR amplification from DNA from clinical fecal samples

The 16S and 23S rDNA from intestinal pathogens were amplified simultaneously directly from fecal samples using asymmetric PCR. All the fecal samples tested gave PCR products with bands of approximately 500 bp and 640 bp (Figure 2 shows partial PCR amplification results for intestinal pathogens from fecal samples).

Validation of the bacterial reference strains using the improved RDB method

The PCR products were used to hybridize with the oligonucleotide probes on the membrane, followed by signal acquisition using the TMB to generate the respective hybridization maps. The results are shown in Figure 3. A given isolate was easily identified as one of the target pathogens from the hybridization signals of the probe spot. The results were in close agreement with those predicted from the layout of the probes. For instance, in the hybridization map shown in Figure 3, array A, there were strong hybridization signals at the

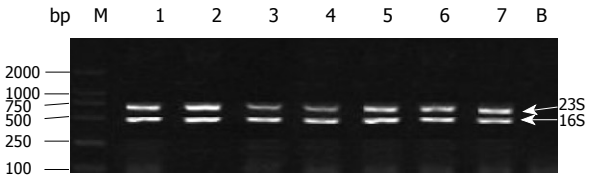

Figure 2 Partial dual PCR amplification results for intestinal pathogens from fecal samples. M: DNA marker 2000; B: Blank control.

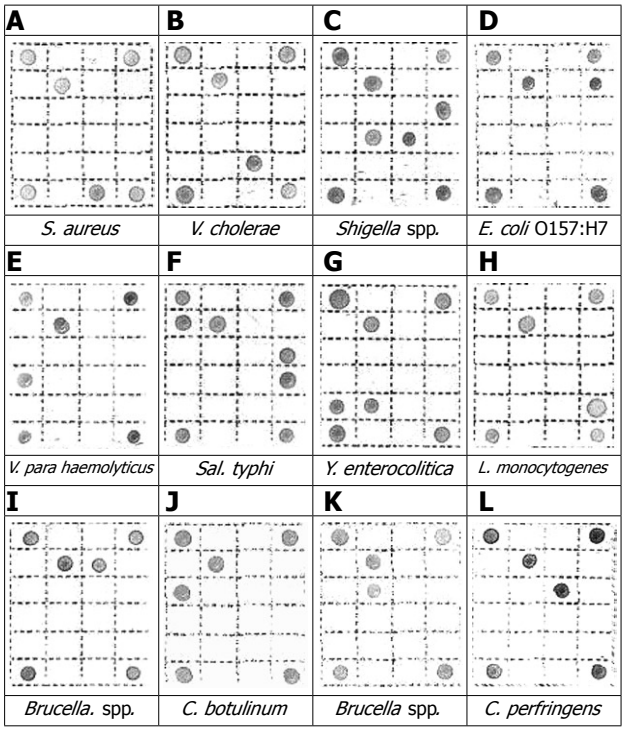

Figure 3 Typical hybridization profiles on the membrane from pure bacterial culture.

sites that corresponded to oligonucleotide probes 17, therefore, the pathogen was sequentially identified as *S. aureus*. Based on the results of multiple experiments, we regarded a hybridization signal as specific if the foreground signal at an oligonucleotide probe site was a stronger color than its background signal. It was easy to identify the specific hybridization signals directly from the hybridization maps by the naked eye. The strains were *Salmonella* spp., *Brucella* spp., *E. coli* O157:H7, *Clostridium botulinum*, *Bacillus cereus*, *Clostridium perfringens*, *Vibrio parahaemolyticus*, *Shigella* spp., *Yersinia enterocolitica*, *Vibrio cholerae*, *Listeria monocytogenes*, and *S. aureus*.

Detection limit of the improved RDB assay

Serial dilutions of a clinical isolate of *E. coli* O157:H7 were tested by using the improved RDB method. The data indicated that as few as 10^3 CFUs could be detected consistently.

Detecting the intestinal pathogens from fecal samples directly

To evaluate the application of this assay, 540 fecal samples from patients with diarrhea were detected.

Table 4 Comparison between the improved RDB and culture

Intestinal pathogen	RDB (+)/ culture (+)	RDB (+)/ culture (-)	RDB (-)/ culture (+)
S. aureus	52	13	12
V. cholerae	4	3	5
Shigella spp.	51	3	3
E. coli O157:H7	28	2	2
V. parahaemolyticus	158	16	11
Salmonella spp.	36	5	3
Y. enterocolitica	3	4	4
L. monocytogenes	4	1	2
B. cereus	3	0	1
C. perfringens	2	0	1
S. aureus and *Shigella* spp.	2	2	0
S. aureus and *Salmonella* spp.	2	0	0
Salmonella spp. and *Shigella* spp.	3	0	0
V. parahaemolyticus and *V. cholerae*	1	0	0
V. parahaemolyticus and *Shigella* spp.	5	1	0
Total	354	50[1]	44[2]

[1] was compared with [2], $\chi^2 = 464$, $P > 0.05$. RDB: Reverse dot blot.

Table 4 compares the results obtained for the intestinal pathogens by a conventional culture and the improved RDB assay. By the improved RDB assay, 404 (74.81%, 404/540) samples were found to be positive for intestinal pathogens, and mixed pathogens were detected from 16 samples. By the culture method, 399 (73.89%, 399/540) samples were found to be positive for pathogens, and mixed pathogens were detected from 13 samples. A total of 354 samples that were RDB positive were also culture positive. Additionally, 50 samples were detected by RDB but were not found by culture. Forty-four of the culture-positive samples were RDB negative. The data indicated that 354 (88.75%, 354/399) specimens identified using RDB were the same as those identified by conventional methods, $\chi^2 = 464$, $P > 0.05$.

DISCUSSION

With the development of more aggressive therapeutic regimens, especially for the treatment of intestinal pathogens, the incidence of foodborne infections has increased. The early initiation of antibacterial treatment is critical in reducing the high mortality rate in patients with infection. Early and accurate identification of the pathogen is the most important and critical step in providing adequate antibacterial therapy in time. The conventional method of identification of intestinal pathogens used in clinical microbiology is based on phenotypic features and physiological tests, and is therefore time-consuming. Instead, molecular genotyping methods could provide a rapid and specific means of identification of intestinal pathogens. At present, diagnostic DNA microarrays are applied for the identification of viruses[18-21], bacteria[22-26], and mechanisms of resistance to certain antibiotics[27-29].

However, the conventional hybridization methods are conducted on two-dimensional surfaces, which require several hours to complete the molecular hybridization

process, and large volumes of sample and reagent. In this study, we described the successful application of the improved RDB method to detect intestinal pathogens. It is a simple, rapid, semiautomatic, reliable, and contamination-proof approach to screen pathogens from fecal samples. We developed a commercially prepared intestinal pathogens detection kit equipped with the KaiPu DNA HybriMax Rapid Hybridization Machine. The machine was designed based on the particular principle of flow-through hybridization. There is a negative pressure under the airproof hybridization membrane that is produced by pumping, so the improved method actively directs the flow of the target molecules toward the immobilized probes within the membrane fibers, which enables rapid hybridization to occur. The dominant characteristic of the improved RDB method is that all of the PCR products, washing buffer, binding solution, and coloring solution flow through the hybrid membrane quickly and directly, with the help of negative pressure, which is semi-automated and is essentially different from the traditional method. The complementary molecules are hybridized and form duplex DNA; at the same time, any unbound molecules are removed through the membrane. This speeds up the interaction between the complementary molecules, reduces the hybridization time from hours down to minutes, and provides results hundreds of times faster than the traditional passive hybridization methods[30].

For the present study, we designed and optimized not only the specific probes for the specific target pathogens, but also the color control probe for the hybridization operation to reach 100% specificity. The color control probe can bind only with the chromogen, but it cannot bind with the target molecule, which helps to judge the validity of hybridization. Instead of using dTTP, we used dUTP and UDG in the PCR system to prevent PCR products from causing contamination. In addition, the improved RDB method is clean, versatile, and less expensive than traditional hybridization. The improved RDB assay directs all of the PCR products and solution to directly flow through the hybrid membrane, which increases the diffusivity and local reaction concentration of the nucleic acid molecule, which occurs in three-dimensional volumes.

We detected 540 fecal samples from patients with diarrhea using the improved RDB assay and culture in parallel. The consistent detection rate of the improved RDB assay compared with the traditional culture method was up to 88.75%. Howerer, the reason that 10 samples were detected by RDB but were not found by culture is that the PCR can amplify DNA fragments even from dead strains, or that the domain bacterial colony grew too rapidly to separate it from the target intestinal pathogens. Otherwise, there is a large amount of unknown substance to disturb the PCR, so that five of the culture-positive samples were RDB negative. However, the data indicated that there was no significant difference between the improved RDB assay and culture to detect the intestinal pathogens from fecal samples. All of these findings indicate that the method is sensitive, specific, and ensures quality in clinical tests.

COMMENTS

Background

Early and accurate identification of the pathogen is the most important and critical step for clinical diagnosis and antimicrobial therapy. Many molecular methods have been applied to pathogen diagnosis.

Innovations and breakthroughs

A new, rapid and accurate reverse dot blot (RDB) method was developed for the detection of intestinal pathogens in fecal samples collected from human subjects, by using the flow-through hybridization principle. The above assay is rapid, accurate stable, reliable and convenient.

Applications

The new technique may play an important role in detection of food poisoning, environmental monitoring, and clinical diagnosis of infectious disease.

Peer review

This is a well performed and interesting study that examined rapid detection methods for intestinal pathogens.

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Magnetic resonance cholangiopancreatography for the detection of pancreatic duct stones in patients with chronic pancreatitis

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Abstract

AIM: To assess the role of magnetic resonance cholangiopancreatography (MRCP) in detection of pancreatic duct stones (PDS) in patients with chronic pancreatitis (CP).

METHODS: Clinical data of 78 CP patients who were treated at the First Affiliated Hospital of Xi'an Jiaotong University (China) between January 2004 and July 2008 were retrospectively analyzed. A predictive model of pancreatic duct stones was established through logistic regression and its effectiveness was verified. Among these patients, MRCP was performed in 60 patients who served as a control group, while 44 patients with a higher predictive value than the entry threshold of the predictive model served as an experimental group.

RESULTS: The positive rate of PDS in the 78 patients with CP was 19.2% (15/78). The predictive entry threshold of the predictive model was 5% ($P < 0.05$). The possibility of existence of PDS could be predicted according to the following 4 indexes: gastrointestinal symptoms, intermittent abdominal pain, diabetes mellitus (DM)/impaired glucose tolerance (IGT) and positive B-mode ultrasound results. The incidence of PDS in the experimental group was higher than that in the control group ($P < 0.05$).

CONCLUSION: MRCP is strongly suggested for the detection of PDS in patients with gastrointestinal symptoms, intermittent abdominal pain, DM/IGT and positive B-mode ultrasound results.

Key words: Chronic pancreatitis; Pancreatic duct stone; Magnetic resonance cholangiopancreatography; B-mode ultrasound; Logistic regression

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INTRODUCTION

Pancreatic duct stone (PDS) is a severe complication of patients with chronic pancreatitis (CP)[1]. It has been reported that 30% of CP patients are complicated by PDS[2]. Conventional examinations are not conclusive for the diagnosis of PDS. To confirm the existence of PDS, imaging examinations, including B-mode ultrasound, computed tomography (CT), endoscopic retrograde cholangiopancreatography (ERCP) and magnetic resonance cholangiopancreatography (MRCP) have been extensively used in the diagnosis of PDS[3-7]. Among them, MRCP is most widely used in clinical practice for the differentiation diagnosis of PDS, pancreatitis, pancreatic tumor, pancreatic cyst and congenital diseases due to its non-invasiveness, high accuracy and repeatability[8-10]. It has been reported that MRCP has a sensitivity of 91.6%, an accuracy of 95.6%, and a specificity of 100% for the detection of PDS[11]. In this study, 78 patients with CP were analyzed retrospectively through a predictive model of PDS based on logistic regression for the rational application of MRCP in detection of PDS.

MATERIALS AND METHODS

Patients

This study included 78 CP patients at the age of 13-66 years (42 males, 36 females, with a median age of 42 years) who were treated at the First Affiliated Hospital of Xi'an Jiaotong University (China) between January 2004 and July 2008. Among them, 60 patients who underwent MRCP served as a control group, while 44 patients with a higher predictive value than the entry threshold of our predictive model served as an experimental group. The study was approved by the Review Board of Xi'an Jiaotong University and written informed consent was obtained from each patient to participate in this study.

Clinical assessment

Clinical data of the 78 patients at admission were analyzed, including gender, age, history of drinking, clinical symptoms (intermittent abdominal pain with or without back pain, progressive emaciation, steatorrhea, cholelithiasis, abdominal distention, anorexia, nausea, vomiting, fever and jaundice), laboratory findings (blood glucose, serum and urine amylase), and the results of B-mode ultrasound examination (pancreatic duct stones or pancreatic duct dilation). The results of MRCP (PHILIPS Intera 1.5T MR scanner) of the 60 patients in experimental group were also collected.

Age over 65 years and less than 65 years was defined as elderly and non-elderly, respectively. History of drinking included alcoholism (> 1 year and > 150 g/d) and non-alcoholism. Hypoglossal temperature over 37.4℃ was defined as fever. Pancreatic duct dilation was diagnosed if the main pancreatic duct diameter was greater than 3 mm in the head and 2 mm in the body or tail of pancreas.

Statistical analysis

Stepwise logistic regression was used to evaluate the factors concerned using SPSS 13.0 for windows. PDS was defined as a dependent variable. "Gender, age, history of drinking, clinical symptoms, laboratory findings and the result of B-mode ultrasound examination" were defined as independent variables. The relative risk was used to express the relation between PDS and each factor (95 % CI). A multivariate logistic regression model was established to obtain the forecasting indexes of PDS. Then a predictive model was established and the prominence of each independent variable was verified with the Wald method.

Primary screening for PDS was performed in the 78 patients using the predictive model. Patients with a higher predictive value than the entry threshold of the predictive model were included in experimental group. Differences in the diagnostic value of MRCP for PDS between experimental and control groups were analyzed by binomial distribution test.

RESULTS

Fifteen of the 78 patients were finally diagnosed with PDS. PDS was found in 10 of the 60 patients who underwent MRCP. The false positive rate of MRCP in the 60 patients was 0% as confirmed at intraoperative examination. Among the other 5 PDS patients diagnosed with B-mode ultrasound or CT (pancreatic dilation or stones) and verified during operation, no PDS was found in 1 at MRCP.

Table 1 Correlation between each factor and PDS

Index	Odds ratio (95% CI)	*P*
Sex	2.47 (1.19, 5.01)	< 0.05
Age	0.54 (0.13, 1.69)	0.95
Drinking history	2.81 (1.25, 6.04)	0.54
Intermittent abdominal pain	6.42 (3.09, 13.72)	< 0.01
Aggravated emaciation	10.62 (4.92, 26.24)	< 0.01
Steatorrhea	1.31 (0.58, 2.78)	0.65
Cholelithiasis	3.54 (1.72, 6.29)	< 0.01
Gastrointestinal symptoms	5.20 (2.63, 12.44)	< 0.01
Fever	0.67 (0.20, 1.98)	< 0.96
Jaundice	9.84 (4.31, 23.47)	< 0.01
DM/IGT	11.24 (6.58, 25.63)	< 0.01
Serum and urine amylase	1.26 (0.53, 2.42)	0.72
Result of B-mode ultrasound	13.64 (6.36, 28.63)	< 0.01

Table 2 Correlation between 4 indexes and PDS

Index	Odds ratio (95% CI)	Wald	*P*
Intermittent abdominal pain	3.83 (1.37, 8.06)	20.23	< 0.05
Gastrointestinal symptoms	4.78 (2.26, 10.14)	23.18	< 0.05
DM/IGT	4.34 (1.85, 8.47)	24.56	< 0.01
The result of B-mode ultrasound examination	12.64 (5.72, 22.39)	29.81	< 0.01

Relation between PDS and indexes

Binary logistic regression revealed that there were a significant relation and a high relative risk between the occurrence of PDS and the 4 indexes including positive result of B-mode ultrasound examination, diabetes mellitus (DM)/impaired glucose tolerance, progressive emaciation and jaundice (Table 1).

Multivariate logistic regression analysis showed that PDS was closely related with the 4 indexes including gastrointestinal symptoms (abdominal distention, anorexia, nausea and vomiting), intermittent abdominal pain, DM/IGT and the positive result of B-mode ultrasound (Table 2). The incidence of PDS was 2%-95% (negative *vs* positive indexes), and increased to 16% when the result of B-mode ultrasound was positive. If the result of B-mode ultrasound was not taken into account, the corresponding incidence of PDS would be 11%-16% for the other two positive indexes and 5%-7% for the single positive index, suggesting that a threshold of 5% could be recommended as the predictive entry threshold for the selection of patients serving as a experimental group (Table 3). The relation between the predictive model and the final diagnosis is shown in Table 4.

Incidence of PDS in different groups

The 44 patients including 15 PDS patients with a

Table 3 Incidence of PDS (%)

Positive indexes(gastrointestinal symptoms, DM/IGT, intermittent abdominal pain)	B-mode ultrasound	
	Negative	Positive
1	5-7	38-41
2	19-23	76-82
3	54	95

higher predictive value than the entry threshold of the predictive model served as the experimental group (Table 4). Considering the high sensitivity and accuracy of MRCP, we assumed that the 4 PDS patients who did not undergo MRCP would have positive results of MRCP, thus increasing the accuracy and reliability of statistical results. Under such conditions, the positive diagnostic rate of MRCP for PDS was significantly different in experimental and control groups (31.8% *vs* 16.7% or 14/44 *vs* 10/60) according to the binomial distribution test.

DISCUSSION

CP, a kind of segmental or diffuse inflammation induced by various causes in pancreatic tissue, presents with recurrent or persistent abdominal pain and progressive dysfunction of pancreas, leading to permanent loss of endocrine and exocrine pancreatic function[12-14]. Early pathological changes in pancreatic tissue include focal fat necrosis, fibrosis of leaflet and duct, and protein thrombus or stones in side branches of the main pancreatic duct. Dilated or obstructed pancreatic duct, eosinophilic protein thrombus and stones can be found in the progressive stage of PDS in the main pancreatic duct.

The incidence of PDS is less than 1% in normal population, while it is about 30% in CP patients[2]. PDS can lead to the damages of pancreatic tissues and corresponding clinical symptoms[15-18]. It was reported that 12%-22.2% of PDS patients finally develop pancreatic adenocarcinoma[19,20]. Therefore, early diagnosis of PDS is of very important clinical significance.

Traditionally, biliary tract disease is the main cause of CP in China[21,22]. With the improvement in living standard, alcoholism is the exceeding biliary tract disease and has become the primary cause of CP. Actually, alcoholism in many countries, especially in developed countries, is the most frequent cause of CP. It has been shown that 70%-80% of CP patients have chronic alcohol drinking history [23-26], and the mortality of alcoholic CP patients has increased to approximately 50% in the past 20 years [25]. However, in our present study, the incidence of alcoholism-related CP (25.8%) was lower than that of biliary tract disease-related CP (38.1%). We assumed that it might be due to the relatively lower living standard in local areas.

In our study, 100% of patients with PDS had abdominal pain, 66.7% had abdominal distention and nausea, 53.3% had DM/IGT. The relation between these clinical manifestations and PDS was confirmed by multivariable logistic regression analysis. On the other hand, as a primary screening method for PDS, B-mode ultrasound has a relatively high sensitivity and specificity. Thus, a predictive model based on the above indexes was established and the predictive entry threshold was set at 5%, and MRCP was recommended as a routine examination for patients with a higher predictive value than the entry threshold.

Table 4 Relation between the prediction model and the final diagnosis

Incidence of PDS in the prediction model (%)	*n*	Final diagnosis		
		Non-stones	Stones	PDS incidence (%)
< 5	34	34	0	0
5-7	26	24	2	7
19-23	5	4	1	20
38-54	2	1	1	50
76-95	11	0	11	100

By analyzing the data through our predictive model, we found that PDS was closely correlated with the 4 indexes (gastrointestinal symptoms, intermittent abdominal pain, DM/IGT and the result of B-mode ultrasound examination). Due to the existence of PDS and calcification, pancreatic duct pressure increases and the pancreatic secretion decreases or loses. Therefore, clinical symptoms such as gastrointestinal symptoms, intermittent abdominal pain, and DM/IGT occur in patients with PDS. Also, direct and/or indirect signs of PDS could be roughly detected by B-ultrasonography. If only one of the indexes is positive, the damage to pancreatic tissue is not serious, and the incidence of PDS is low.

In conclusion, MRCP is strongly recommended for the final diagnosis of PDS in patients with gastrointestinal symptoms, intermittent abdominal pain, DM/IGT and positive B-ultrasonography result.

COMMENTS

Background

Pancreatic duct stone (PDS) is a severe complication of chronic pancreatitis (CP). It was reported that 30% of CP patients are complicated by PDS. At present, the diagnosis of PDS depends on some imaging examinations, such as B-mode ultrasound, computed tomography (CT), endoscopic retrograde cholangiopancreatography (ERCP) and magnetic resonance cholangiopancreatography (MRCP), *etc*.

Research frontiers

Compared with other imaging techniques, MRCP is easy to perform and has no contraindication. It could provide detailed information about pancreatic duct and common bile duct. Therefore, MRCP is widely applied in the diagnosis of PDS.

Innovations and breakthroughs

In this study, a predicative model was established in an attempt to increase the diagnosis rate of PDS by MRCP. The result supports application of MRCP in detection of PDS.

Peer review

The authors established a predictive model for PDS in CP patients and verified that 4 indexes were closely correlated with PDS, which may elevate the diagnosis rate of PDS.

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CASE REPORT

Cecal volvulus: Report of a case and review of Japanese literature

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Abstract

A 78-year-old woman presented with fever, severe abdominal pain, and distension. She had been institutionalized for depression and senile dementia. Laboratory examinations disclosed a leucocytosis (WBC: 12 500/μL) and elevated levels of serum C-reactive protein (2.8 mEq/L). Diagnosis of acute cecal volvulus was made from a "coffee bean sign" on an abdominal computed tomography and a "beak sign" on a gastrographin enema. An emergent laparotomy confirmed the diagnosis and an ileo-colectomy with primary anastomosis was carried out. The patient recovered after intensive respiratory care and fluid therapy, and then returned to her former institution. A review of Japanese literature disclosed that: (1) a marked increase of aged patients with mental disability presenting with cecal volvulus, (2) adoption of ileo-colectomy as the standard surgical procedure, and (3) improved survival of the patients, were observed in the last decade.

Key words: Cecal volvulus; Ileo-colectomy; Mental disability

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INTRODUCTION

Cecal volvulus is axial twisting that occurs involving the cecum, terminal ileum, and ascending colon. Rarely, it may take the form of upward and anterior folding of the ascending colon ("cecal bascule")[1]. Cecal volvulus is a rare condition, and its incidence is reported to range from 2.8 to 7.1 per million people per year[1].

Clinical presentation is highly variable, ranging from intermittent episodes of abdominal pain to abdominal catastrophe[2,3]. In this paper, we report a case of cecal volvulus seen in a 78-year-old woman. Ages at presentation, causative factors, treatment and outcome of 40 cases reported in Japan between 1999 and 2008 were also reviewed, and compared with those in cases reported before 1988[4].

CASE REPORT

A 78-year-old woman was admitted with acute abdominal pain and distension. She had an operation for gastric cancer at the age of 73, and had been institutionalized for depression and dementia.

On admission, she had a high fever of 38.8℃. Her abdomen was diffusely distended with rebound tenderness. Laboratory examinations disclosed a leucocytosis (WBC: 12 500/μL) and elevated serum C-reactive protein levels (2.8 mEQ/L).

Plain radiographs of the abdomen showed a markedly dilated loop of the intestine, occupying most of the abdomen, but the pathology was not certain. A "coffee-bean sign", on an abdominal CT (Figure 1), together with "a beak sign" on a gastrogrephin enema (Figure 2), confirmed the diagnosis of cecal volvulus[3,5].

An emergent laparotomy disclosed the axial twisting of the cecum, involving the terminal ileum and the ascending colon. Ischemia of the bowel seemed to be irreversible, but there was no perforation (Figure 3).

An ileo-colectomy with primary anastomosis was

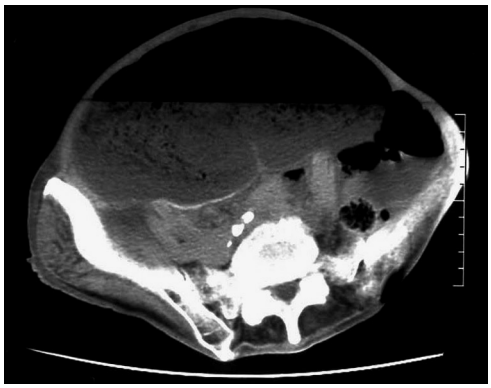

Figure 1 A "coffee-bean sign" on an abdominal CT film.

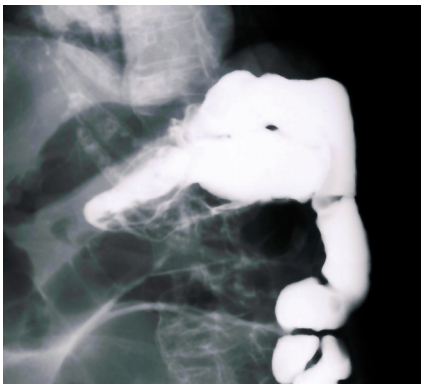

Figure 2 A "beak sign" on a gastrographin-enema.

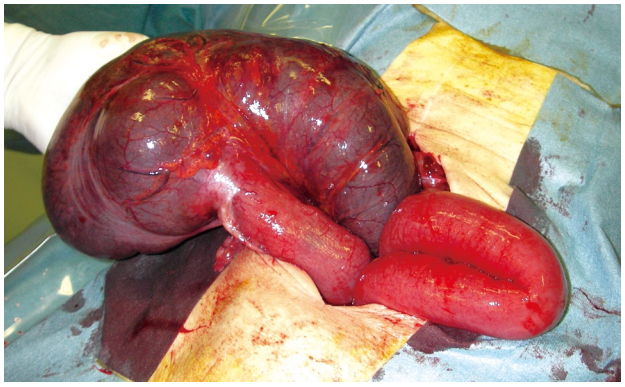

Figure 3 Operative finding after detorsion of axial torsion of the right colon. Ischemia of the bowel seemed to be irreversible, but there was no perforation.

carried out. Postoperatively, the patient was placed under close monitoring and managed by an intensive respiratory care and fluid therapy until stabilized. Then she was discharged and returned to her former institution.

DISCUSSION

The incidence of cecal volvulus is reported to range from 2.8 to 7.1 per million people per year[1]. Patients' ages at presentation, the treatment of choice, and survival differ by reports chronologically as well as geographically[2,3].

We compared ages at presentation, treatment of choice, and outcome of 46 patients reported in Japan before 1988[4] with reports of 40 patients collected from a search of Japan Centra Revus Medicina in the last 10 years.

Patients' age at the presentation are said to be affected by cultural and dietary influences. Gupta and Gupta[6] reported that the average age at presentation in Western countries was 53 years, whereas it was 33 years in India. In Japan, patients treated before 1988 showed two peaks in ages at presentation; one in the 10-29 years range and another in the 60-79 years range. On the other hand, there was only one peak in ages of the patients treated between 1999 and 2008; 70-89 years of age (Table 1). In the young age group, the volvulus was caused by mesenterium commune, other intestinal malformation, or excessive exercise. In contrast, volvulus in the aged patients was associated with chronic constipation, distal colon obstruction, or senile dementia.

Table 1 Ages at the presentation of cecal volvulus

Age	Patients treated before 1988	Patients treated between 1999 and 2008
0 to 9	3	1
10 to 19	9	4
20 to 29	5	3
30 to 39	5	2
40 to 49	3	2
50 to 59	4	3
60 to 69	10	3
70 to 79	50	12
80 to 89	2	8
Over 80	0	2

χ^2-test: $P = 0.008$.

Table 2 Treatment of cecal volvulus

	Patients treated before 1988	Patients treated 1999 to 2008
Endoscopic detorsion	0	4
Surgery	45	36
Detorsion	9	0
Cecopexy	14	8
Cecostomy	7	0
Ileo-Colectomy	15	28
Unknown	1	0

χ^2-test: $P < 0.0001$.

Surgical options include cecopexy, cecostomy, and ileo-colectomy by open or laparoscopic approaches. Review of Japanese literature disclosed that resectional surgery was done in 32.6% (15/46) of patients who were treated before 1988, whereas 70% (28/40) of patients underwent ileo-colectomy between 1999 and 2008 (Table 2). This significant increase of the patients treated by resectional surgery ($P < 0.0001$ by χ^2 test) was brought about by advances in surgical techniques and perioperative supportive measures.

Flexible colonoscopy is commonly used for the diagnosis and the treatment of sigmoid volvulus, but the utility of endoscopy in acute cecal volvulus is limited,

because of technical problems and higher rates of recurrence[1]. Our review also showed that reports on colonoscopic detorsion are just emerging (Table 2).

Mortality, morbidity, and recurrence rates were also lower in recent years. O'Mara *et al*[7] reported in 1979 that they treated 14 patients with cecal volvulus by ileo-colectomy and lost 2 of 7 patients who developed gangrenous bowel, while there were no deaths amongst 7 patients with non-gangrenous bowel. Gupta *et al*[6] reported that they encountered operative death in 2 of 13 patients who underwent resectional surgery. However, Majeski *et al*[8] reported in 2005 that there was no operative deaths in 10 patients treated by ileo-colectomy. Our review of Japanese literature disclosed that the outcome after surgical treatment has markedly improved in the last 10 years. Before1988, there were five postoperative death in 45 patients who underwent surgery (15 resectional, and 30 non-resectional), whereas only one of 36 patients treated surgically (8 resectional and 28 non-resectional) between 1999 and 2008 was lost.

As the mortality and the morbidity after resectional surgery for acute cecal volvulus, whether gangrenous or non-gangrenous, have been markedly improved and almost no recurrence is observed, an ileo-colectomy with primary anastomosis should be the preferred surgical option rather than cecopexy or a cecostomy.

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CASE REPORT

A rare case of bile duct cyst

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Abstract

Choledochal cyst is an uncommon disease usually seen in young women and can be divided into five types. We report a 66-year-old woman who was diagnosed with types Ⅰ and Ⅲ bile duct cyst simultaneously after surgery, which is a rare type of bile duct cyst.

Key words: Choledochal cyst; Common bile duct; Cholangiography

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INTRODUCTION

Choledochal cyst (CDC) is a rare type of bile duct cyst of uncertain origin, which was first described and classified by Todani *et al*[1] in 1977. The majority of cases reported are young women and children of Asian descent, especially in Japan and Taiwan. The most common symptoms of CDC are abdominal pain, jaundice and abdominal mass. We present a rare case of an elderly female patient with CDC.

CASE REPORT

A 66-year-old woman was admitted to our hospital because of epigastric pain and dark urine for 10 d. She had some fatty foods before the onset of pain and received antibiotic treatment in a local hospital. She had a 10-year medical history of abdominal pain, which was related to her high-fat diet. She was diagnosed with cholecystitis and quickly recovered after antibiotic treatment.

At physical examination, her blood pressure was 140/90 mmHg, her pulse was 80 beats/min, her body temperature was 37℃, her respiratory rate was 18 breaths/min, and her skin and sclera were yellow-stained. She had tenderness in the epigastric region, and was positive for Murphy's sign.

Laboratory tests revealed 18.84 × 10^9/L white blood cells, 89.8% granulocytes, 983 IU/L (0-115 IU/L) blood amylase, 158.4 μmol/L total bilirubin and 130.23 μmol/L direct bilirubin.

Abdominal ultrasound showed that the diameter of her common bile duct was 1.3 cm, her intra-hepatic bile duct was dilated, and her gallbladder was enlarged. A primary diagnosis was thus made of acute biliary pancreatitis, obstructive jaundice and acute cholecystitis.

The patient received routine treatment for pancreatitis, and her clinical symptoms and objective signs improved, and her biochemical parameters subsequently returned to their normal levels. However, an abdominal computer tomography (CT) scan suggested that her common bile duct and pancreatic duct were dilated and her pancreatic head was enlarged (Figure 1). Endoscopic examination showed a cystic tumor connected to the papilla in the duodenum (Figure 2). An upper gastrointestinal contrast scan showed a filling defect in the horizontal part of the duodenum with a long pedicel in the intestinal canal (Figure 3).

The patient underwent an operation after consultation. The dilated common bile duct was like a shuttle, and a cyst was observed in the horizontal part of the duodenum connected to the duodenal papilla. Bile leakage was found at excision of the cyst. The cyst was completely removed. Cholecystectomy was performed with reconstruction by Roux-en-Y choledochojejunostomy. The patient recovered quickly and was discharged 20 d after surgery.

DISCUSSION

CDC, also known as congenital common bile duct cyst

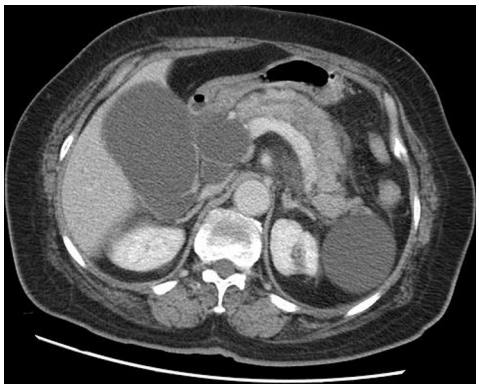

Figure 1 Abdominal CT revealing the dilated common bile duct and pancreatic duct and enlarged pancreatic head.

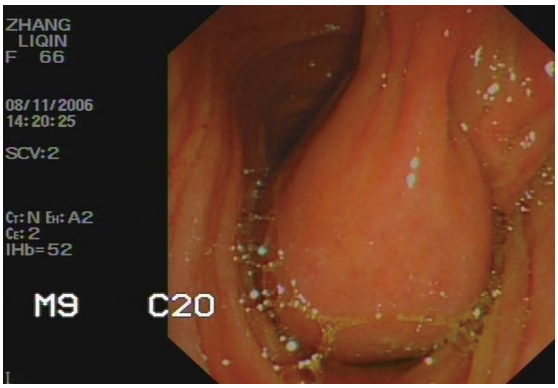

Figure 2 Endoscopy showing a cystic tumor connected to the papilla in duodenum.

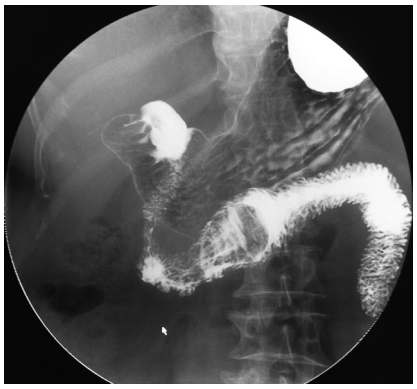

Figure 3 Upper gastrointestinal contrast scan displaying a filling defect in the horizontal part of the duodenum.

(BDC), is a rare type of bile duct cyst of uncertain origin. The majority of cases reported are young women and children of Asian descent. In North America, its incidence is estimated to be 1/150 000[2], but it is increasing in Western adults. The most common symptoms of CDC are abdominal pain, jaundice and abdominal mass.

CDC is graded based on Todani's classification[1] and can be divided into five types, which are anomalies in the intra- or extra-hepatic bile ducts, or both. CDC can be diagnosed based on percutaneous and endoscopic ultrasound or endoscopic retrograde cholangiopancreatography. Magnetic resonance cholangiography may also contribute to its diagnosis.

Type Ⅳ cysts are more commonly observed in adults than in children[3], while type Ⅰ cysts are more commonly observed in Asian patients[4]. Type Ⅲ cyst, a cystic-like dilation of the terminal common bile duct, is rare[5,6]. Our patient had a CDC, consistent with types Ⅰ and Ⅲ grading. To the best of our knowledge, this is the first case of bile duct cyst in the English-language literature. Total excision of the diseased bile duct with reconstruction by Roux-en-Y choledochojejunostomy is a preferred treatment modality for CDC[7]. However, its long-term effects on CDC need to be proven in follow-up.

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CASE REPORT

Combined *en bloc* liver/pancreas transplantation in two different patients

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Abstract

Combined *en bloc* liver/pancreas transplantation (CLPT) was used primarily in the treatment of otherwise non-resectable upper abdominal malignancy. In fact, a more appropriate indication is in patients with liver disease and insulin-dependent diabetes mellitus (IDDM). Here, we report on two successful cases of CLPT at our hospital. One was a patient with non-resectable advanced liver cancer. The recipient survived for 23 mo and finally died of recurrent tumor. The other was a patient with severe biliary complication after orthotopic liver transplantation and preoperative IDDM. We performed CLPT with a modified surgical technique of preserving the native pancreas. He is currently liver-disease- and insulin-free more than 27 mo post-transplant. Based on our experience in two cases of abdominal cluster transplantation, we describe the technical details of CLPT and a modification of the surgical procedure.

Key words: Transplantation; Liver; Pancreas; Diabetes mellitus; Liver cancer

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INTRODUCTION

Although originally described several decades ago, combined liver/pancreas transplantation (CLPT) is still a relatively uncommon procedure. There are relatively few indications for CLPT. Previously, it was used mostly as a lifesaving method in the treatment of otherwise non-resectable upper abdominal malignancies[1,2]. However, with poor results, mainly because of tumor recurrence, this procedure fell out of favor. In fact, a more ideal indication for this so-called abdominal organ cluster transplantation is in patients with liver disease and insulin-dependent diabetes mellitus (IDDM). However, there have been only six case reports of successful CLPT in this group of patients[3,4], including three children[5].

Starzl originally designed the operation with removal and replacement of the entire grape cluster. During the procedure, upper abdominal exenteration (the liver, pancreas, spleen, duodenum, part of the stomach) is carried out. Exenteration is necessary for treatment of abdominal malignancies. However, in patients with liver disease and IDDM, such massive abdominal evisceration is unnecessary.

With the increased practicality of multivisceral transplantation, different innovative techniques have been introduced to further improve survival and reduce morbidity. Fishbein and Abu-Elmagd recently presented their experiences on preservation of the native organs in patients with hepatic-intestinal, and isolated intestinal transplantation[6,7]. According to Starzl, the main subtypes of multivisceral transplantation are full multivisceral, upper abdominal (cluster), hepatic-intestinal, and isolated intestinal transplantation[8]. In this paper, we present our experience of preservation of the native organs in a patient who underwent upper abdominal (cluster) transplantation. We compare this patient to another one with advanced liver cancer who underwent well-described standard CLPT.

CASE REPORT

Case 1

Patient 1 is a 44-year-old man (blood group B^+; 68 kg; 174 cm) who presented with a 12-mo history of discomfort and vague pain in the right upper abdomen. Ultrasonic examination and follow-up computed tomography (CT) showed a lesion measuring 5 cm × 5 cm in the right lobe of the liver, portal vein embolus, and enlarged pancreatic head. The level of alpha-fetoprotein (AFP), serum alanine transaminase (ALT), and total serum bilirubin (T-Bil) was 1096 μg/L, 104 IU/L and 22.7 μmol/L respectively. The patient had a 20-year history of hepatitis B. He was diagnosed with advanced liver cancer. Multivisceral transplantation was an alternative that might have offered the only chance of radical tumor excision.

In September 2004, ABO compatible size matched organs from a 48-year-old 62-kg male donor were allocated. At laparotomy, pancreatic infiltration and embolus invading the portal vein and superior mesenteric vein were confirmed. After complete removal of the recipient liver, duodenum, part of the stomach and small bowel, CLPT was performed *en bloc* with the conventional technique. The grafts were sewn in to the native inferior vena cava in a standard fashion. Then, the superior mesenteric vein of the graft was anastomosed to the native one. Finally, a donor aortic patch with the celiac trunk and the superior mesenteric artery was anastomosed end-to-end to the receptor common hepatic artery. After reperfusion of the graft, the gastrointestinal tract was reestablished with a Roux-en-Y duodenojejunostomy (Figure 1). The operative time, amount of blood loss and blood transfusion during the operation were 600 min, 7 L and 32 units, respectively.

Immunosuppression included induction with daclizumab and maintenance treatment with the triple immunosuppressive therapy (tacrolimus, mycophenolate mofetil and prednisolone). His liver function recovered fast. On the seventh day after operation, the grafts test showed AFP, ALT, T-Bil were < 10 μg/L, 84 IU/L and 12.7 μmol/L, respectively. Blood sugars remained normal post-transplant. His postoperative course was complicated by severe mixed pulmonary infections on postoperative day 7, which lasted for 3 wk. Furthermore, intra-abdominal hemorrhage occurred on postoperative day 16, which required re-operation. He undertook six courses of postoperative chemotherapy with a pirarubicin-containing regimen. The recipient was doing well until ultrasound and CT showed hypodense areas in his new liver one and a half years post-operation. He finally died of cancer recurrence 23 mo post-operation (Table 1).

Case 2

In 2006, a 49-year-old man (blood group A^+; 63 kg; 173 cm) presented with severe jaundice and high fever (39℃). He had a history of liver transplantation for terminal hepatitis-B cirrhosis in 2002. Unfortunately, severe biliary complication developed postoperatively. Since then, he suffered from very severe jaundice and repeated high fever with biliary infection. Meanwhile, he had a medical history of IDDM since about age 10 years. His blood sugar became difficult to control after liver transplantation. Furthermore, blurred vision occurred, which was finally diagnosed as diabetic ophthalmopathy.

Table 1 Patient characteristics

Characteristics	Patient 1	Patient 2
Age (yr)	44	49
Gender	Male	Male
Indication	Malignancy	Liver disease and DM
During operation		
Operative time	600 min	570 min
Amount of blood loss	7000 mL	3000 mL
Blood transfusion	32 units	13 units
Postoperative complications		
Pulmonary infections	+	-
Intra-abdominal hemorrhage	+	-
Outcome	Dead (23 mo postoperation)	Alive (27 mo posttransplant)

In September 2006, the patient underwent CLPT with organs from a 25-year-old male donor (blood group A^+; 60 kg; 171 cm). Removal of the native organs was relatively simple. We only needed to resect the pathological liver, and then the organ cluster was transplanted orthotopically. A piggy-back anastomosis of the grafted suprahepatic vena cava onto the native one was performed. Then, the grafted superior mesenteric vein was anastomosed to the native portal vein. Next, a circular donor aortic patch including the celiac trunk and superior mesenteric artery was anastomosed end-to-end to a donor aortic tube that had been previously implanted on the receptor infrarenal aorta. The pancreas graft was draped directly over the native pancreas. The time of the anhepatic phase was 56 min. Finally, the digestive tract was reconstructed. A Roux-en-Y anastomosis of the grafted distal duodenum and the native proximal jejunum was performed (Figure 2). The operative time, amount of blood loss and blood transfusion during the operation were 570 min, 3 L and 13 units, respectively.

The patient experienced an uneventful postoperative recovery. Since the second day post-operation, he no longer needed exogenous insulin. Liver function also recovered rapidly. On the first day after the operation, the grafts test showed that ALT and T-Bil was 531 U/L and 169.8 μmol/L, respectively. On postoperative day 7, they declined to 44 U/L and 63 μmol/L. Two weeks after transplantation, his liver function became normal. He is currently alive, liver-disease- and insulin-free more than 27 mo post-transplant (Table 1).

DISCUSSION

The operation of *en bloc* CLPT stemmed from the pioneer work of Thomas E. Starzl more than four decades ago[9]. Starzl and Williams published their first successful clinical experience in 1989[10,11]. Their initial attempts proved that the technique was feasible. However, because of postoperative complications and technical problems, the complex procedure is now still

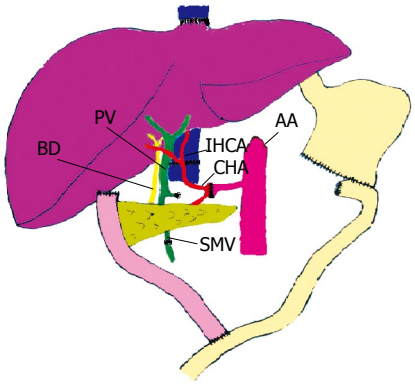

Figure 1 Standard *en bloc* CLPT. The grafts were sewn into the native inferior vena cava. The superior mesenteric vein (SMV) of the graft was anastomosed to the native one. A donor aortic patch with the celiac trunk and the superior mesenteric artery was anastomosed end-to-end to the receptor common hepatic artery (CHA). The gastrointestinal tract was reestablished with a Roux-en-Y duodenojejunostomy. IHCA: Infrahepatic cava anastomosis; BD: Donor bile duct; PV: Donor portal vein; AA: Native abdominal aorta.

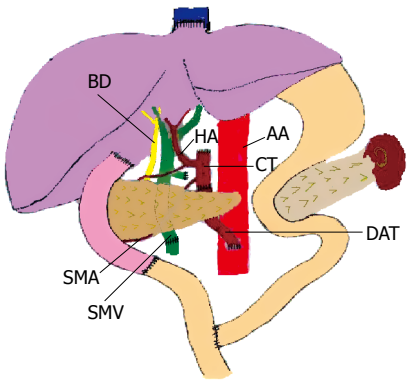

Figure 2 Modified *en bloc* CLPT while retaining the native pancreas. A piggy-back anastomosis of the grafted suprahepatic vena cava onto the native one was performed. A circular donor aortic patch including both the celiac trunk (CT) and the superior mesenteric artery (SMA) was anastomosed end-to-end to a donor aortic tube (DAT) that had been implanted previously on the native abdominal aorta (AA). The donor superior mesenteric vein (SMV) was anastomosed end-to-end to the native portal vein. Roux-Y anastomosis of the grafted distal duodenum and the native proximal jejunum was performed. HA: Donor hepatic artery; BD: Donor bile duct.

performed in small numbers in a few transplant centers.

There are relatively few indications for CLPT. Previously, it has been mostly indicated in patients with otherwise non-resectable upper abdominal malignancies[1,2]. Although some of the patients with advanced malignancies could benefit from this radical operative approach, it is not a wise choice to allocate scarce donor organs to such patients. There are several barriers to improving patient survival.

First, postoperative hemorrhage is one of the common life-threatening complications after such massive abdominal evisceration. Two of the first four recipients reported by Starzl and Williams succumbed from uncontrollable bleeding shortly after the complex surgery. Secondly, infection is a barrier to the improvement of graft survival[12], and represents the leading cause of mortality[13]. Thirdly, tumor recurrence remains the most difficult barrier to improving patient and graft survival. All three of these complications occurred in our patient 1.

In fact, a more appropriate indication for this so-called abdominal organ cluster transplant is in liver transplant candidates who coincidentally suffer from IDDM. For them, we only need to perform hepatectomy before transplanting the *en bloc* liver-duodeno-pancreatic graft. Case 2 showed the success of the surgical modification, while retaining the native pancreas. We believe that there are several potential advantages of such a modified technique.

First, the pancreas is located deep in the abdomen with an abundant blood supply. Avoiding native pancreas removal means avoiding surgical damage to the surrounding tissues. This procedure can dramatically decrease oozing of blood into the surgical field, particularly in patients with severe adhesions in the upper abdomen. Secondly, retaining the native pancreas endocrine and exocrine secretion can relieve the burden on the new pancreas. Thirdly, the gastrointestinal tract remains as a complete reunification of the whole, with retention of the native normal duodenum. Thus, the recipient can start feeding early postoperatively. Early feeding can prevent bacterial translocation and villus atrophy[14]. Fourthly, the recipient spleen is not removed during this procedure. Previous studies have revealed that the asplenic state is associated with increased incidence of sepsis[15]. Finally, avoiding removal of the native greater omentum may help to reduce postoperative complications, especially in cases of anastomotic leakage and duodenal or pancreatic fistula.

In conclusion, our experience demonstrates that *en bloc* CLPT can be modified according to the patient's need. Although it plays a role as rescue therapy, the procedure for advanced abdominal malignancy needs careful consideration. The experience so far supports further cautious trials with this drastic cancer operation. A more appropriate indication is in patients with terminal benign liver disease and IDDM. We consider that the modified technique that preserves the native pancreas has the potential to become the standard procedure for this group of patients.

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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
London, UK
Gastro 2009 UEGW/World Congress of Gastroenterology
www.gastro2009.org

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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Instructions to authors

GENERAL INFORMATION

World Journal of Gastroenterology (*World J Gastroenterol* ISSN 1007-9327 CN 14-1219/R) is a weekly open-access (OA) peer-reviewed journal supported by an editorial board consisting of 1179 experts in gastroenterology and hepatology from 60 countries.

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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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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EDITORIAL

Human herpesvirus 6 infections after liver transplantation

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Abstract

Human herpesvirus 6 (HHV-6) infections occur in > 95% of humans. Primary infection, which occurs in early childhood as an asymptomatic illness or manifested clinically as roseola infantum, leads to a state of subclinical viral persistence and latency. Reactivation of latent HHV-6 is common after liver transplantation, possibly induced and facilitated by allograft rejection and immunosuppressive therapy. Since the vast majority of humans harbor the virus in a latent state, HHV-6 infections after liver transplantation are believed to be mostly due to endogenous reactivation or superinfection (reactivation in the transplanted organ). In a minority of cases, however, primary HHV-6 infection may occur when an HHV-6 negative individual receives a liver allograft from an HHV-6 positive donor. The vast majority of documented HHV-6 infections after liver transplantation are asymptomatic. In a minority of cases, HHV-6 has been implicated as a cause of febrile illness with rash and myelosuppression, hepatitis, pneumonitis, and encephalitis after liver transplantation. In addition, HHV-6 has been associated with a variety of indirect effects such as allograft rejection, and increased predisposition and severity of other infections including cytomegalovirus (CMV), hepatitis C virus, and opportunistic fungi. Because of the uncommon nature of the clinical illnesses directly attributed to HHV-6, there is currently no recommended HHV-6-specific approach to prevention. However, ganciclovir and valganciclovir, which are primarily intended for the prevention of CMV disease, are also active against HHV-6 and may prevent its reactivation after transplantation. The treatment of established HHV-6 disease is usually with intravenous ganciclovir, cidofovir, or foscarnet, complemented by reduction in the degree of immunosuppression. This article reviews the current advances in the pathogenesis, clinical diagnosis, and therapeutic modalities against HHV6 in the setting of liver transplantation.

Key words: Immunocompromised; Antivirals; Human herpesvirus 6; Liver transplantation; Opportunistic infections

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INTRODUCTION

Human herpesvirus 6 (HHV-6), a member of the β-Herpesviridae subfamily of human herpesviruses, is a ubiquitous virus that was first isolated from peripheral blood leukocytes in 1986[1]. The virus was not associated with any clinical illness until 2 years later, when HHV-6 was isolated from the peripheral blood of patients with roseola infantum (also known as exanthem subitum or sixth disease), which is a common febrile illness in children[2]. Since then, there have been large-scale epidemiologic studies that have established the natural history of HHV-6 infections in humans. Primary infection with HHV-6 occurs most commonly during the first 2 years of life, with a peak incidence between 6 and 12 mo after birth. By 2 years, more than 90% of individuals have been infected, as evidenced by a positive HHV-6 antibody. Primary HHV-6 infections may present as an asymptomatic illness or as a febrile syndrome accompanied later on by a maculopapular rash (exanthem subitum). In addition, primary HHV-6 infection has been associated with otitis, gastrointestinal symptoms, respiratory distress, and seizures[3,4].

There are two variants of HHV-6, variant A and variant B (HHV-6A and HHV-6B, respectively). These variants share certain biological properties and have a high level of sequence homology, differing from one another only by up to 8% at the nucleotide level[5,6]. However, the two HHV-6 variants differ epidemiologically and clinically. HHV-6B is implicated in the majority of primary HHV-6 infections during the first 2 years of life. HHV-6B replicates in the salivary glands[7] and hence, the mechanism of transmission between humans is thought to be via salivary secretions. In contrast, HHV-6A seems to be more neurotropic and has been implicated in neurologic diseases[8,9]. HHV-6A is also more frequently detected among patients with acquired immunodeficiency syndrome[10]. The age of acquisition of HHV-6A remains undetermined and, unlike HHV-6B, it does not seem to replicate in salivary glands and thus, the mode of transmission is not known.

PATHOPHYSIOLOGY

HHV-6 infects mainly CD4+ T lymphocytes, and to a lesser extent, CD8+ T lymphocytes and natural killer cells[11-13]. HHV-6 binds to the CD46 receptor[14] that is located on what is called a "lipid raft," which then carries the virus inside the cell. The HHV-6 envelope fuses to the cell membrane, and the viral nucleocapsid is transported into the nucleoplasm where the viral DNA genome is released[15]. The virus then replicates, assembles, and exits the infected cell to infect other cells. The first proteins synthesized during viral replication are the immediate-early (IE) proteins[16]. It was recently reported that two proteins, IE1 and IE2, distinguish the variants, HHV-6A and HHV-6B, respectively (L. Flamand, abstract 3-2, 6th International Conference on HHV6 & 7, 2008). However, the exact mechanism of HHV-6 replication and assembly is not clear, although viral assembly occurs inside multivesicular bodies which are subsequently transported toward the cell surface to facilitate virus exit from the cell.

One of the mechanisms postulated to explain the ability of HHV-6 to escape the immune system and establish latency is its property of immunomodulation. HHV-6 infection results in altered cytokine responses resulting in the selective suppression of interferon-γ[17], interleukin-2[18] and up-regulation of tumor necrosis factor-α production[19]. In addition, HHV6 down-regulates the expression of its CD46 receptor[12,14], which functions as a regulator of complement activation[20] and an important link between the innate and adaptive arms of the immune system. Finally, HHV-6 has been demonstrated to enhance apoptosis *in vitro*[21,22].

LATENCY AND CHROMOSOMALLY-INTEGRATED HHV-6

After primary infection, HHV-6 establishes a state of subclinical persistence or latency. This is a property that it shares with the other members of the human herpes virus family. During latency, the HHV-6 genome is harbored as separate circular DNA inside various cells, such as lymphocytes and probably monocytes. HHV-6 DNA sequences have been detected in peripheral blood mononuclear cells of as many as 90% of one study population[23].

In rare cases, instead of existing as a separate circular DNA, HHV-6 integrates into a human chromosome [termed chromosomally-integrated HHV-6 (CIHHV-6)]. Both HHV-6A and HHV-6B have been found to have this ability to become integrated into the chromosome. The incidence of CIHHV-6 is not exactly known, although a recent study of blood donors from the United Kingdom estimated an incidence between 0.2%-1%[24]. It is suggested that CIHHV-6 may also be vertically transmitted (mother to child transmission)[25], since it was found in germ cell lines, however, this has not been confirmed by other investigators[26]. Individuals with CIHHV-6 have a characteristic persistently high levels of HHV-6 DNA in the blood, sera, and hair follicles, without causing clinical illness[27]. Individuals with CIHHV-6 persistently have high levels of HHV-6 DNA in the blood, usually millions of genomic copies, while patients with acute HHV-6 infection or reactivation only have viral copies in the tens of thousands, even in the setting of immune compromise. A reliable method which can be used to distinguish CIHHV-6 is a quantitative PCR of a hair follicle sample, which often is negative in non-CIHHV-6 infections. The high level of DNA found in the blood and other body fluids in CIHHV-6 is due to cellular proliferation and cellular lysis and not as a result of viral replication. The clinical significance of CIHHV-6 is not clear. While many believe this is not related to significant clinical problems, there are few reports suggesting that CIHHV-6 may be associated with an increased risk of lymphoproliferative disease[28,29].

MECHANISMS OF HHV-6 INFECTION AFTER TRANSPLANTATION

Because the vast majority of humans harbor latent HHV-6, infection with this virus after transplantation is believed to result from viral reactivation. Viral reactivation may also occur in the transplanted allograft to cause HHV-6 superinfection in a previously-infected individual. In a minority of cases, primary HHV-6 infection may occur in a transplant recipient through the allograft or blood products, or through natural transmission (e.g. exposure to oropharyngeal secretions). Primary HHV-6 infection is likely more common in the pediatric transplant population, especially in children less than 2 years of age, who have not been exposed to infection. In this very young group of patients, there is a higher likelihood of receiving an allograft or blood products from a previously infected donor.

INCIDENCE OF AND RISK FACTORS FOR HHV-6 INFECTION AFTER LIVER TRANSPLANTATION

The incidence of HHV-6 infection after liver

transplantation has been reported to range between 14% and 82%[30-32]. HHV-6 infections typically occur during the first 2-8 wk after liver transplantation when the level of immunosuppression is most intense. However, HHV-6 infections as early as 10 d and as late as 5 years after liver transplantation have been reported[33]. Since the vast majority of patients have developed HHV-6 infections during early life and harbor the latent virus, the vast majority of HHV-6 infections after transplantation are believed to be due to endogenous reactivation. Factors that have been associated with HHV-6 reactivation after liver transplantation are acute allograft rejection and receipt of high doses of corticosteroids[33,34]. The presence of HHV-6 infection in cases of acute liver failure has also been reported as a risk factor for the development of allograft hepatitis after liver transplantation[35].

CLINICAL SYNDROMES ASSOCIATED WITH HHV-6 INFECTION AFTER LIVER TRANSPLANTATION

A myriad of clinical syndromes have been associated with HHV-6 infection after liver transplantation. These have been classified as either direct or indirect effects of HHV-6. The direct clinical manifestations due to HHV-6 include a febrile illness with or without rash, myelosuppression, hepatitis, pneumonitis and neurological diseases[33,36-38]. The indirect effects attributed to HHV-6 include an exacerbation of cytomegalovirus (CMV) disease, an increased severity of hepatitis C virus (HCV) recurrence, an increased risk of other opportunistic infections, allograft dysfunction, and acute cellular rejection (Table 1)[33,36,39-45].

Table 1 Clinical syndromes attributed to HHV-6 after liver transplantation

HHV-6 direct effects	Ref.	HHV-6 indirect effects	Ref.
Fever and rash	[36]	Increased incidence and severity of cytomegalovirus disease	[31,40,41]
Hepatitis	[33,36,49]	Earlier and more severe recurrence of hepatitis C virus	[42]
Myelosuppression	[37]	Higher incidence of fungal infections	[44]
Pneumonitis	[37]	Higher incidence of opportunistic Infection	[36]
Neurologic illness	[38,44,51]	Higher incidence of allograft rejection	[33,36,45,53]

HHV-6: Human herpesvirus 6.

Direct HHV-6 effects

Fever and rash: The most frequently reported clinical presentation of HHV-6 infection after liver transplantation is a febrile illness that can be associated with a rash[34,36,46]. In a study of 200 liver transplant recipients, two patients (1%) presented with a febrile illness and HHV-6 was implicated as the causative agent, after excluding all other pathogens or etiologies of the fever[36]. In many cases, this febrile illness may clinically mimic, and thus be misdiagnosed as, CMV syndrome[47]. Thus, it has been suggested that the syndrome of febrile illness with myelosuppression and rash after transplantation be termed as β-herpesvirus syndrome while the specific viral etiology is being investigated[48] Co-infections with HHV-6 and CMV have been demonstrated in these cases[47]. However, a recent large study of solid organ transplant recipients demonstrated that HHV-6 was not significantly associated with any clinical symptoms during CMV disease[41].

Hepatitis: HHV-6 has been implicated as a cause of hepatitis after liver transplantation. In a review of 121 patients who developed hepatitis after liver transplantation, 8 (6.7%) cases were thought to be secondary to HHV-6 infection[33], as documented by serology and immunoperoxidase staining of liver biopsy specimens. Clinically, HHV-6 infection was associated with elevated liver enzymes, allograft dysfunction, acute rejection, and lymphocytic infiltration. These clinical findings were also observed in another report of one patient who had lymphocytic infiltration and elevated aminotransferases during HHV-6 infection[36]. In another report, an HHV-6B infected transplant recipient developed syncytial giant cell hepatitis as a result of donor-transmitted HHV6-A infection[49]. Serologic, molecular, and immunohistochemical methods were used to identify HHV-6A superinfection as the etiologic agent in this patient with a latent HHV-6B infection[49]. Finally, another study showed that nine of 18 patients who had pre-transplant HHV-6 infection developed HHV-6 hepatitis after liver transplantation[50].

Myelosuppression and pneumonitis: Bone marrow suppression is another clinical presentation attributed to HHV-6 infection. In a report of four liver transplant recipients, HHV-6 associated myelosuppression occurred at a median of 50 d (range 17-90 d) after liver transplantation. While all the cell lineages were affected, leukopenia was the most common presentation. One of the four patients in this report also had concomitant interstitial HHV-6 pneumonitis, as documented by a positive HHV-6 immunostaining of the lung biopsy[37].

Neurological illness: Encephalitis due to HHV-6 infection has been reported in two liver transplant recipients[38,51]. In another report, central nervous system complications such as mental status changes of unidentified etiology were more likely to occur in liver transplant recipients who had HHV-6 infection[44]. However, another report found no significant association between HHV-6 infection and neurological illnesses[36]. These contradictory results may be due to the differences in neurotropism between HHV6 variants, with HHV-6A as the neurotropic variant. These differences in clinical manifestations should be considered in the analysis of the clinical impact of HHV-6 after liver transplantation.

Indirect HHV-6 Effects

Impact on CMV disease: HHV-6 is postulated to have immunomodulating properties that enhance the reactivation of CMV. Alternatively, the presence of HHV-6 may serve as a marker of an over-immunosuppressed state and hence the predisposition to develop other infections such as CMV. In one study, liver transplant recipients with documented primary HHV-6 seroconversion had a higher incidence of symptomatic CMV disease compared to those who did not have HHV-6 seroconversion[39]. This finding was again demonstrated in a prospective study wherein liver transplant recipients who developed CMV disease had detectable HHV-6 DNA in the blood[31]. Recently, a retrospective study showed that 16 of 19 liver transplant recipients who developed symptomatic CMV infection had concomitant HHV-6 antigenemia, including 12 patients who developed HHV-6 infection prior to CMV antigenemia[40]. However, this association between HHV-6 and CMV was not observed in a large cohort of solid organ transplant recipients who received oral ganciclovir and valganciclovir prophylaxis, wherein the incidence of CMV disease was not significantly different between those who develop and those who did not develop HHV-6 DNAemia[41].

Impact on HCV disease: A prospective study reported that HCV-positive patients who developed HHV-6 viremia after liver transplantation had an earlier recurrence and a higher fibrosis score upon hepatitis C recurrence when compared to patients without HHV6 viremia[42]. In another analysis of 60 liver transplant recipients with chronic hepatitis C, HHV-6 infection did not influence the incidence of hepatitis C recurrence, but was associated with more severe hepatitis and a higher fibrosis score[43]. In contrast, a study of 93 hepatitis C infected liver transplant recipients showed no association between HHV-6 and the incidence and severity of hepatitis C recurrence after transplantation[52].

Impact on fungal and other opportunistic infections: Because of its immunomodulating properties, HHV-6 has been postulated to influence the occurrence of other opportunistic infections after liver transplantation. In one study of 200 liver transplant recipients, the impact of HHV-6 infection on opportunistic infections, including CMV, Epstein Barr virus-related post-transplant lymphoproliferative disease, varicella zoster virus, invasive fungal infections, and mycobacterial disease, was demonstrated. In a multivariate analysis, HHV-6 was found to be a significant risk factor for the occurrence of these opportunistic infections[36]. In another study, HHV-6 was independently associated with invasive fungal infections in a cohort of 80 liver transplant recipients[44]. Similarly, in a study of 247 patients, the incidence of invasive fungal infection was 2-fold higher in patients with HHV-6 seroconversion compared to those without HHV-6 seroconversion[36]. It was further demonstrated that HHV-6 infection was an independent predictor of invasive fungal infections during the first 90 d after liver transplantation[36]. Whether this is due to the immunomodulating properties of the virus, or whether this is due to an over-immunosuppressed state (with HHV-6 reactivation as a marker of over-immunosuppression) remains to be defined.

Impact on allograft rejection and function: The association between HHV-6 and allograft dysfunction and rejection has been demonstrated in a few studies[33,36]. Local HHV-6 infection in the allograft was associated with increased expression of adhesion molecules on vascular endothelial cells and infiltrating leukocytes, and this could lead to local inflammation and graft damage leading to dysfunction and possible rejection[53]. In an analysis of liver transplant recipients who developed allograft rejection, HHV-6 infection and peak HHV-6 load were the only factors significantly associated with rejection beyond 30 d after liver transplantation[36]. Another study further supported the independent association between HHV-6 and biopsy-proven acute allograft rejection after liver transplantation[45]. However, these associations remain debatable since treatment for allograft rejection may also lead to HHV-6 reactivation. Hence, the association between HHV-6 and allograft rejection may be bidirectional.

DIAGNOSIS OF HHV-6 INFECTION

Distinguishing HHV-6 reactivation (i.e. active replication) from latency can be challenging because of the highly prevalent nature of latent HHV-6 infection in humans. Over 95% of adults have been exposed to the virus and express antibodies against HHV-6. The various assays used to diagnose active HHV-6 infection are summarized in Table 2.

Real time polymerase chain reaction (PCR)

Molecular assays are the most commonly used laboratory methods to detect HHV-6 reactivation and replication after transplantation. Both quantitative and qualitative methods have been developed to detect HHV-6 DNA in blood and clinical samples[54-57]. In addition to blood samples, HHV-6 detection by PCR can be performed on biopsy and tissue specimens[58]. These assays can differentiate between the variants HHV-6A and HHV-6B as a result of base-differences[54]. PCR testing has some limitations, mainly due to the inability of most assays to distinguish latent from replicating virus. To address this, it is suggested that serum samples are used, since the virus is cell-associated and the detection of free viral particles in cell-free serum would be more indicative of active HHV-6 infection[54]. This is not the case for whole blood specimens where latent HHV-6 may be present and amplified from leukocytes. The use of quantitative PCR assays may be helpful in distinguishing replicating from latent HHV-6, with the premise that high HHV-6 levels or increasing viral levels over time would indicate true HHV-6 replication[55]. In this context, it is emphasized that one may rarely detect the presence of CIHHV-6, as discussed above, so that high levels (often

Table 2 Tests for the laboratory diagnosis of HHV-6 infection

Test	Detects active infection	Distinguish HHV-6 variants A and B	Commercially available
Serology (IFA and ELISA)	No	No	Yes
Real time PCR			
Qualitative	No	Yes	Yes[1]
Quantitative	Yes	Yes	Yes[1]
Culture	Yes	Yes	Yes[2]
Real time reverse transcriptase PCR	Yes	Yes	No[3]
Antigen testing	Yes	Yes	No
Antibody avidity testing	Yes	No	No
Immunohistochemical technique	Yes	Yes	No
PCR *in situ*	Yes	Yes	No

IFA: Immunofluorescence assay; ELISA: Enzyme-linked immunosorbent assay; PCR: Polymerase chain reaction; [1]Available at reference and commercial laboratories; [2]Requires viral stimulation by chemicals; [3]Very specialized research laboratories.

in the million copies) in CIHHV-6 infected individuals do not necessarily reflect active viral replication. The detection of HHV-6 RNA by real time reverse transcriptase PCR assay, on the other hand, would indicate the presence of actively replicating virus[57].

Serology

Serologic assays by immunofluorescence assay (IFA) or enzyme-linked immunosorbent assay (ELISA) are commercially available to detect antibodies against HHV-6 in plasma and serum samples. However, these assays cannot differentiate between HHV-6A and HHV-6B variants. IFA seems to be more useful as titers can be followed over time to demonstrate a rise or fall in antibody levels. In contrast, ELISA cannot be used to compare the index value over time. Both IgM and IgG can be measured. HHV-6 IgM increases during the first few weeks after infection and will be detected for several months thereafter. However, the clinical utility of serology testing after transplantation is often questionable since the results are not uncommonly false-negative due to the inability of immunosuppressed patients to develop antibodies. Nonetheless, the presence of HHV-6 IgM antibodies confirms a primary infection. HHV-6 IgG antibodies are detected initially several weeks after primary infection, and they remain elevated during latent infection. In transplant recipients, elevated IgG level has been used to suggest HHV-6 reactivation, although this is of questionable significance. An antibody avidity test may differentiate recent and past infection[59]. Some patients, particularly those receiving potent immunosuppressive therapy, may not be able to generate antibodies, and hence, using HHV-6 serology alone may miss an acute infection or reactivation.

Culture

Culture is not widely available for the detection of HHV-6 for various reasons. It is time consuming, expensive, and the virus is difficult to grow unless it is activated by chemicals. Moreover, growth of the virus does not necessarily distinguish latency from active growth *in vivo*[37,60,61].

Table 3 Antiviral molecules and their activity against HHV-6

Antiviral drug	*In vitro* activity	*In vivo* activity[1]	Mechanism of antiviral resistance
Acyclovir[73,81]	No	No	
Ganciclovir[66-68,70-72]	Yes	Yes	Mutation in U38 DNA polymerase Mutation in U69 phosphotransferase
Foscarnet[65,82-85]	Yes	Yes[2]	Mutation in U38 DNA polymerase
Cidofovir[76,86,95]	Yes	Yes	Mutation in U38 DNA polymerase
Maribavir[87,88]	No	Not available	
Cyclopropavir[89]	Yes	Not available	
CMV 423[91]	Yes[3]	Not available	
HDP-CDV[90]	Yes[3]	Not available	
3 Deaza-HPMPA[92]	Yes	Not available	

CMV 423: A New anti-CMV (Cytomegalovirus) molecule (2-chloro 3-pyridine 3-yl 5,6,7,8-tetrahydroindolizine 1-carboxamide); HDP-CDV: Hexadecyloxopropyl-cidofovir; 3 Deaza-HPMPA: (S)-9-[5-Hydroxy-2-(phosphonomethoxy) propyl]-3 deazaadenosine. [1]Based on very limited studies and case reports; [2]Combination of foscarnet with ganciclovir or cidofovir has been reported and can be efficacious; [3]Activity *in vitro* demonstrated against HHV6-A.

Antigen testing

HHV6 antigenemia can be detected in whole blood samples or tissue specimens using specific monoclonal antibodies. It often indicates the presence of an active infection, and may distinguish variant HHV-6A from HHV-6B. However, this technique is labor-intensive, semi-quantitative, and it is not widely available for clinical use[55,62].

PREVENTION AND TREATMENT OF HHV-6 INFECTIONS

There have been no randomized clinical trials conducted on antiviral drugs for the prevention and treatment of HHV-6 disease in humans. As a result, there is currently no antiviral drug that is FDA-approved for clinical use in HHV-6 infection. Nonetheless, ganciclovir, cidofovir and foscarnet have been used in the clinical setting for the treatment of HHV-6 associated diseases, although the potential efficacy of these drugs have been based mainly on *in vitro* experimental data and on anecdotal case reports (Table 3).

Acyclic nucleoside analogues (Ganciclovir)

Ganciclovir is the most commonly used drug for the management of HHV-6 infections. However, this use is not supported by randomized controlled clinical trials. Ganciclovir inhibits viral DNA polymerase, which functions during viral replication. For it to exert its antiviral properties, ganciclovir must undergo tri-

phosphorylation into the active metabolite, ganciclovir-triphosphate. The initial phosphorylation requires the enzyme phosphotransferase, which is expressed by HHV-6[63]. *In vitro* studies demonstrated the activity of ganciclovir against HHV-6[64,65]. Clinically, ganciclovir has been shown to be effective in HHV-6 infected bone marrow transplant recipients[66-70]. Likewise, case reports suggest that ganciclovir is effective for the treatment of HHV-6 infections after liver transplantation[66-68,70-72]. Ganciclovir prophylaxis has been shown to be effective in preventing HHV-6 reactivation in stem cell transplant recipients[73-75]. However, some cases of fulminant HHV-6 infections may not respond to ganciclovir[68,69]. The inconsistencies in these reports could be due to the differential susceptibilities to ganciclovir between variants HHV-6A and HHV-6B. Studies have demonstrated that HHV-6B is less susceptible to ganciclovir when compared to HHV-6A[76,77]. In the clinical setting, this differential susceptibility could partly explain the occurrence of HHV-6B (but not HHV-6A) infections in a large cohort of solid organ transplant recipients who received anti-CMV prophylaxis with ganciclovir or valganciclovir[41]. In addition, HHV-6 isolates that are resistant to ganciclovir have been described. This is due to mutations in the U38 DNA polymerase or the U69 phosphotransferase genes[78-80]. Unlike ganciclovir, acyclovir appears to be ineffective against HHV-6 clinically and *in vitro*[81]. Acyclovir prophylaxis was not effective in preventing HHV-6 reactivation in stem cell transplant recipients[73-75].

Foscarnet (phosphonoformic acid)

Foscarnet is a pyrophosphate analogue which inhibits viral replication by targeting viral DNA polymerase. Foscarnet has been shown to be active against HHV-6 *in vivo* and *in vitro*[65,82,83]. The combination of foscarnet with ganciclovir or cidofovir was also shown to be efficacious based on a case report[84]. *In vitro* studies showed that mutation in the DNA polymerase would render HHV-6 resistant to foscarnet[85].

Cidofovir

Cidofovir is an acyclic nucleoside phosphonate analogue that has been shown to have excellent activity against HHV-6 *in vitro*[76]. There have been clinical reports where cidofovir was used successfully to treat HHV-6 infections. However, cidofovir is considered a second line treatment because of its nephrotoxicity. A mutation in the U38 gene encoding DNA polymerase was found to be responsible for a mutant HHV-6 that is highly resistant to cidofovir[86].

Investigational agents

Although several agents being developed for the treatment of viral pathogens do not specifically target HHV-6, some have been tested for their activity against this pathogen. Maribavir, a benzimidazole derivative that is being developed for the management of CMV infection, was demonstrated to be inactive against HHV-6 *in vitro*[87,88]. Recently, the clinical development of maribavir for the prevention of primary CMV disease after liver transplantation has been terminated since it was not demonstrated to be superior to placebo in stem cell transplant recipients. On the other hand, cyclopropavir, a recently developed guanine nucleoside analogue, has been shown to have activity against HHV-6 *in vitro*[89]. Hexadecyloxopropyl-cidofovir, a prodrug of cidofovir, has also been shown to be three times more potent than cidofovir against DNA viruses, including HHV6-A[90]. CMV 423, a new anti-CMV molecule (2-chloro 3-pyridine 3-yl 5,6,7,8-tetrahydroindolizine 1-carboxamide) that inhibits tyrosine kinases, likewise has been shown to have good activity against HHV-6A[91]. 3 Deaza-HPMPA[(S)-9-(5-Hydroxy-2-(phosphonomethoxy) propyl)-3 deazaadenosine] has been shown to be 6-fold more active than cidofovir *in vitro* against HHV-6A and HHV-6B[92]. Finally, arysulfone derivatives[93] and artesunate[94] seem to have some activity against HHV-6. However, the clinical development of these investigational drugs is at an early stage and it is not clear on whether they will eventually reach the bedside.

CONCLUSION

Subclinical HHV-6 infections in immunocompromised transplant recipients are common, while clinical HHV-6 disease is uncommon. Indeed, some have even suggested that detection of HHV-6 infection after liver transplantation may just serve as a virologic marker of an over-immunosuppressed status. Nonetheless, some of the reported HHV-6 associated diseases have led to serious complications and even mortality. The immunomodulatory effect of HHV-6, particularly its interaction with other viruses, and its effect on allograft survival in liver transplant recipients are very intriguing and need to be further elucidated. Hence, a better understanding of the pathobiology of HHV-6 in liver transplant recipients is needed. This goal, however, is hampered by the challenges in clinical diagnosis due to the lack of standardized diagnostic methodologies. Although currently-available antivirals have been used to treat severe cases of HHV-6 infections, well-controlled clinical studies that support the use are lacking. Novel anti-herpetic agents under development have been shown to exhibit activity against HHV-6 *in vitro*, but data on their efficacy in the clinical setting is lacking and need to be assessed in future clinical studies.

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TOPIC HIGHLIGHT

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Nutritional status and nutritional therapy in inflammatory bowel diseases

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Abstract

Underweight and specific nutrient deficiencies are frequent in adult patients with inflammatory bowel disease (IBD). In addition, a significant number of children with IBD, especially Crohn's disease (CD) have impaired linear growth. Nutrition has an important role in the management of IBD. In adults with CD, enteral nutrition (EN) is effective in inducing clinical remission of IBD, although it is less efficient than corticosteroids. Exclusive EN is an established primary therapy for pediatric CD. Limited data suggests that EN is as efficient as corticosteroids for induction of remission. Additional advantages of nutritional therapy are control of inflammation, mucosal healing, positive benefits to growth and overall nutritional status with minimal adverse effects. The available evidence suggests that supplementary EN may be effective also for maintenance of remission in CD. More studies are needed to confirm these findings. However, EN supplementation could be considered as an alternative or as an adjunct to maintenance drug therapy in CD. EN does not have a primary therapeutic role in ulcerative colitis. Specific compositions of enteral diets-elemental diets or diets containing specific components-were not shown to have any advantage over standard polymeric diets and their place in the treatment of CD or UC need further evaluation. Recent theories suggest that diet may be implicated in the etiology of IBD, however there are no proven dietary approaches to reduce the risk of developing IBD.

INTRODUCTION

Inflammatory bowel disease (IBD), Crohn's disease (CD), ulcerative colitis (UC) and indeterminate colitis are chronic inflammatory disorders of the gastrointestinal tract. The relationship between nutrition and IBD is complex and involves several aspects. These include: (1) nutritional support for malnourished patients, (2) primary therapy for active disease and maintenance of remission and (3) nutrients as risk factors involved in the etiology of IBD.

Nutritional care is important in the treatment of patients with IBD and includes prevention or treatment of malnutrition and micronutrient deficiencies, prevention of osteoporosis, and, in children, promotion of optimal growth and development. Enteral nutrition (EN) is considered the modality of choice for the treatment of active CD in children and for some adults too.

NUTRITIONAL STATUS OF ADULT PATIENTS WITH IBD

Malnutrition is common in patients with IBD, especially in active CD. Several studies have documented weight loss in 70%-80% of hospitalized IBD patients and in 20%-40% of outpatients with CD[1,2]. The prevalence of malnutrition is lower in patients with UC, but nutritional deficiencies can develop fast in these patients during periods of active disease[3].

Table 1 Pathophysiology of malnutrition

Main mechanism	Effect
Decreased food intake	Anorexia
	Abdominal pain, nausea, vomiting
	Restricted diets
	Drugs
Nutrients malabsorption	Reduced absorptive surface due to inflammation, resection, bypass and fistulae
Increased intestinal loss	Exudative enteropathy (protein loss)
	Occult/overt blood loss (iron deficiency)
	Diarrhea (increased loss of Zn^{2+}, K^{+}, Mg^{2+})
	Steatorrhea (fat and fat soluble vitamin malabsorption, and divalent cations' loss: Zn^{2+}, Mg^{2+}, Ca^{2+}, Cu^{2+})
Hypermetabolic state	Alterations of resting energy expenditure
Drugs' interaction	Anorexia, nausea, test alteration, proteolysis, interaction with nutrients absorption/utilization

Pathophysiology of malnutrition

The main mechanisms responsible for malnutrition in CD are presented in Table 1. These may cause malnutrition either alone or in combination. Factors which have a major role in one nutritional deficiency may play a minor role in the appearance of a different deficiency in the same patient. The most important causes of malnutrition are probably reduced food intake[4,5], presence of active inflammation[6] and enteric loss of nutrients during periods of disease activity but also during remission[7]. Anorexia secondary to increased levels of proinflammatory cytokines [tumor necrosis factor-α, interleukin (IL)-1 and IL-6][8], white adipose tissue adipokines (leptin, adiponectin, resistin)[9] and suspected alterations in hypothalamic serotonin levels[10] are considered the main causes of reduced food intake.

Studies on energy metabolism in patients with CD have been contradictory. Energy expenditure has been reported to be increased, normal, or even reduced in IBD patients compared with healthy individuals[11,12]. This may be partly because patients with different disease extension, inflammatory activity, and nutritional status were grouped together. However, when adjusted for body composition, increased resting energy expenditure (REE) has generally been disclosed. Furthermore, despite being malnourished, children with CD fail to adapt their REE per unit of lean body mass, an additional factor contributing to malnutrition[13,14].

NUTRITIONAL ASSESSMENT OF PATIENTS WITH IBD

A variety of nutritional and functional deficiencies have been observed in patients with active or inactive CD. The prevalence of malnutrition had decreased as awareness rose, and recent studies showed that most patients in remission are in a good nutritional status and some are even overweight, but still have significant abnormalities in body composition. Sousa Guerreiro *et al*[15] reported that the BMI of CD patients overall was lower than of controls

Table 2 Nutritional assessment in patients with IBD

Assessment	Parameters	Percentage of CD patients with deficient intake or parameters
Dietary history	Energy intake, low	40%[5,15]
	Protein intake, high	150% RDA[5]
	Carbohydrates, excess	39.2%[17]
	Fat, and saturated fat, excess	27%, and 59.5%[17]
	Iron intake, low	50%, 13%[18]
	Calcium and phosphor intake, low	23%[18]
	Folate intake, low	19%[18]
	Vitamin A intake, low	13%-21%, 26%[18,19]
	Vitamin B intake, low	18%-37%[19]
	Vitamin C intake, low	21%-34%, 11%[18,19]
	Vitamin D	36%[18]
	Vitamin E	63%[18]
Anthropometry	IBW < 90%	40%[5]
	BMI > 25 kg/m^2	32%[15]
Body composition	Fat body mass, SFT < 15%	30%[5]
	Fat free mass, MAC < 15%	59%[5]
	DXA (dual-energy X-ray absorptiometry)	30% osteopenic, 60% sarcopenic[20]
	Nitrogen balance, negative	[21]

RDA: Recommended daily allowance; IBW: Ideal body weight; BMI: Body mass index; SFT: Skinfold thickness triceps; MAC: Mid arm circumference.

($P = 0.006$). Thirty two percent of patients with CD had BMI > 25 kg/m^2, but still had lower fat free mass and significantly lower adjusted mean daily intakes of carbohydrates, monounsaturated fat, fiber, calcium, and vitamins C, D, E, and K ($P < 0.05$). Muscle mass depletion was detected in more than half of CD and UC patients even in the absence of malnutrition. BMI, arm muscle area and triceps plus subscapular skin fold thickness values were significantly lower, but only in the active phase of CD[3]. Valentini *et al*[16] evaluated in a prospective, controlled, multicenter study, the nutritional status, body composition, muscle strength, and quality of life in patients with IBD in clinical remission. They showed that, despite most being well nourished (74%), both CD and UC patients have decreased body cell mass and handgrip strength (as a functional measure of nutritional status) when compared to controls. This shows that the most prevalent form of malnutrition in CD patients has changed to one of excess body weight, coupled with inadequate dietary intake of micronutrients, secondary to dietary exclusion of certain foods. Moreover, in spite of appropriate intakes of energy and macronutrients, CD patients in remission have significantly lower plasma concentrations of several vitamins and minerals (Tables 2 and 3)[17-21].

NUTRITIONAL STATUS AND GROWTH IN CHILDREN WITH IBD

Growth failure and malnutrition are one of the major complications affecting children with IBD. Weight loss is present at diagnosis in up to 90% of children[22]. Recent studies have shown that, similar to adults, a significant proportion of children with CD are overweight. In a

Table 3 Nutritional deficiencies in patients with IBD

Macro- and micro-nutrient deficiencies	Nutrients	Percentage of CD patients with deficiencies
Hypoproteinemia and hypoalbuminemia		17.6[18]
Anemia	Iron deficiency	39.2[18]
	B12 deficiency	18.4[18]
	Folic acid deficiency	19[18]
Electrolytes and trace elements	Zinc	15.2, 65[18,19]
	Copper	84[19]
	Selenium	82[19]
Vitamins' deficiency (low serum levels)	B12 deficiency	18.4[18]
	Vitamin A	23.4[18]
	Vitamin B	29[18]
	Vitamin C	84[19]
	Vitamin D	17.6[18]
	Vitamin E	16[15]

Table 4 Pathophysiology of growth failure in children with IBD

Ethiopathogenesis	Mechanism
Energy and nutrient deficiencies[30]	Deficits of energy, macronutrients and micronutrients
Inflammation/proinflammatory cytokines[31]	Anorexigenic effect
	GH-IGF1 axis effects
	Bone metabolism disturbance
	Hypermetabolic/catabolic effects
Disease severity and disease location[32,33]	Severe disease
	Jejunal localization
Abnormal bone metabolism[34]	Effect of pro-inflammatory cytokines
	GH-IGF1 axis dysfunction
	Calcium and vitamin D deficiency
	Delayed sexual maturation
	Corticosteroids
Delayed onset of sexual maturation[35]	Hypogonadism
Abnormal IGF1 axis[36]	Low IGF1 and IGF1-BP
	Proinflammatory cytokines
Drugs[28]	Corticosteroids

cohort of 783 patients with newly diagnosed IBD, low BMI (< 5%) was seen in 22%-24% of children with CD and 7%-9% of children with UC. Ten percent of children with CD and 20%-30% of children with UC had a BMI consistent with overweight or risk for overweight[23]. Despite their preserved fat mass, children with CD frequently have low lean body mass[24]. Growth retardation at diagnosis has been reported in 23%-88% of children with CD and may precede the gastrointestinal manifestations by years[25]. Growth failure is less common in UC compared to CD although growth impairment is seen in both groups[26]. The variability in reported prevalence of growth failure in children with CD can be explained by differences in the definition of growth impairment, the population under study and disease phenotype (colon *vs* small bowel). About 30%-40% of children continue to have severe linear growth retardation during their disease course and several studies found that the final height is affected in CD patients with early onset symptoms[27,28]. The etiology of growth failure is multi factorial and not completely understood, but poor nutritional state, systemic consequences of gut inflammation, disturbances of the growth hormone/insulin-like growth factor axis, genetic influences and corticosteroid use contribute in different ways (Table 4)[29-36].

INTERVENTION FOR GROWTH IN CHILDREN WITH CD

Newby *et al*[37] examined the results of different interventions for growth failure in children with CD. Three randomized, controlled trials (RCT) were identified. One study looked at the use of 6-mercaptopurine (6-MP) as a steroid sparing agent[38]. No difference in linear growth was observed between the intervention and placebo groups, although the total steroid dose received over the 18 mo follow-up period was reduced in the group receiving 6-MP. Two other trials compared EN to corticosteroids for induction of remission. In both studies, height velocity standard deviation scores were significantly increased in the EN group compared with the corticosteroid group[39,40]. The judicious use of surgical interventions was also shown to improve growth in pre-pubertal children with refractory disease[41-43]. In a large prospective trial of infliximab in children and adolescents with moderate to severe CD, improvement of height velocity and height percentiles was seen in children treated with the drug prior to or early during puberty[44]. The role of growth hormone for the treatment of growth failure associated with CD is unclear. The few studies that investigated the effect of growth hormone on growth velocity showed contradictory results and the effect of such treatment on final adult height is yet to be determined[45,46].

NUTRITION AS PRIMARY THERAPY FOR ADULTS WITH CD

Parenteral nutrition (PN)

Dudrick *et al*[47] were the first to suggest that PN was safe and possibly beneficial to patients with IBD. Use of PN for the management of adults with CD during the eighties succeeded in achieving clinical remission and avoiding surgery[47,48]. However, the remission was often short lived and the number of patients remaining in remission 3 mo later varied between 20% and 79% depending on the population of patients, length of PN administration, definitions of remission or recurrence and simultaneous use of medications[49]. PN therapy also achieved fistula healing in 43%-63% of patients in some series, accompanied by reduction in disease activity index, weight gain and elevation of serum albumin[50,51]. PN was proved a useful adjunctive therapy for UC patients requiring bowel rest and nutritional support, though not useful in induction of remission[52].

Since EN was shown to be at least as efficient as

PN with lower costs and fewer significant side effects, the current indications for PN support are restricted to severe malnutrition and for nutritional support pre- and postoperatively, in both CD and UC[53,54].

Home parenteral nutrition

CD accounts for up to 20% of the adult population on home PN[55,56]. PN has an important role in maintaining the nutritional status and improving the quality of life of these patients, but it is associated with significant morbidity and potentially life-threatening complications. In a retrospective series of 41 patients on home PN for CD over an 11-year period (121 patient-years of home PN), 58.5% of patients had one or more PN-related complications necessitating hospitalization. There were eight deaths, one directly caused by catheter-related sepsis[57].

Experience in children using home PN is limited. Strobel *et al*[58] have reported their experience in 17 pediatric patients, all of whom had severe CD. All 17 patients showed weight gain and symptomatic improvement and 10 had height catch up. Complete remission was obtained in 12 patients during the first course of PN[58].

Enteral nutrition for adult patients with CD

EN was shown to induce clinical remission, improve nutritional status, improve body composition, induce mucosal healing, decrease pro-inflammatory cytokine levels and reduce serum inflammatory markers in patients with CD[59-63]. The theory behind the mechanism of action of EN is multi-factorial (Table 5)[64-67].

Three meta-analyses and two Cochrane Database Systematic Reviews published in recent years examined the efficacy of EN compared to corticosteroids in CD[68-72]. The most recent Cochrane meta-analysis that included 192 patients treated with EN and 160 treated with steroids yielded a pooled OR of 0.33 favoring steroid therapy (95% CI: 0.21-0.53). In patients in whom remission was achieved, the relapse rates at 12 mo were identical (65% and 67%) regardless of the therapy. Similar results were reported in all meta-analyses of adult patients. It must be remembered, however, that since meta-analyses are based on intention to treat analysis, they also reflect the lower acceptance of this form of treatment in adults. Furthermore, comparison of efficacy alone between EN and corticosteroids is insufficient, as the two treatment modalities possess entirely different safety profiles. In contrast to corticosteroids, EN has minor, immediate side effects and no known long-term adverse effects. Adult patients should be considered also for EN if: (1) there is a potential for a high lifetime corticosteroid dose, including adolescents and patients in their thirties; (2) there is a high risk for osteoporosis; (3) the patients are steroid-refractory, steroid-dependent or steroid-intolerant; (4) the patients request alternative treatment.

Type and content of formula

Elemental formulae (protein provided as amino acids) were utilized in the initial studies in adults with IBD. Studies comparing elemental formulae to polymeric (whole protein) drinks showed that the two formulae were equally efficacious. Similar conclusions were reached by the Cochrane Database Systematic review that examined one form of EN *versus* another for inducing remission of active CD. Meta-analysis of 10 trials comprising 334 patients found no difference in the efficacy of elemental *versus* non-elemental formulae (OR 1.10, 95% CI: 0.69-1.75). Subgroup analyses performed to evaluate the different types of elemental and non-elemental diets (elemental, semi-elemental and polymeric) showed no significant differences[72]. The reviewers concluded that protein type does not influence the effectiveness of EN.

Table 5 The mechanism of action of enteral nutrition in CD

Proposed mechanism of action	Ref.
Improvement of nutritional status	[59]
Down regulation of pro-inflammatory cytokines	[64,65]
Anti-inflammatory effects	[61,62]
Promote epithelial healing	[62,65]
Decrease gut permeability	[66]
Decrease antigenic load to the gut, bowel rest	[59]
Modification of gut flora	[67]

The influence of fat quantity and quality of enteral diets on the outcome in CD has been examined in several studies. The use of diets with a very low fat content (0.6%-1.3% of total calories) has been associated with good outcomes[73], while those containing high quantities of fat (12%-30% of total calories) were associated overall with less favorable outcomes, in particular when large amounts of linoleic acid were present[74,75]. The 2007 Cochrane Database Systematic review examined seven trials (209 patients), treated with EN formulae of differing fat content (low fat: < 20 g/1000 kcal *versus* high fat: > 20 g/1000 kcal), and found no significant difference in efficacy between the 2 (OR 1.13, 95% CI: 0.63-2.01). Similarly, the effect of very low fat content (< 3 g/1000 kcal) or type of fat (long chain triglycerides) did not demonstrate a difference in efficacy in active CD, although a non significant trend favoring very low fat and very low long chain triglyceride content was demonstrated[72].

Different modifications in composition of enteral formulae have also been evaluated. Such modifications include fat and/or protein content as described, and the addition of bioactive peptides such as glutamine, growth factors (transforming growth factor-β2), butyrate, omega-3 fatty acids and antioxidants[61,76-79]. Addition of bioactive peptides to enteral diet formulae may have specific anabolic or anti-inflammatory actions. Up to now, such modifications of enteral diets-elemental diets or diets containing specific components-have not been shown to have any advantage over standard polymeric diets.

EN and disease location in adults with either CD or UC

In patients with CD, disease location was not found to predict induction of remission with EN. Zachos *et al*[72] concluded, after extensive review of the literature, that a definite statement about the impact of disease location upon response to EN cannot be made because of

insufficient data. There is no evidence to support the use of EN as primary therapy in UC.

EN FOR MAINTENANCE OF REMISSION IN ADULTS WITH CD

Ongoing EN supplementation may help maintain remission and reduce the use of corticosteroids. When using this strategy, supplementary oral formula is provided in combination with a normal diet throughout the day. This approach may also be used in combination with maintenance medical therapy.

The Cochrane IBD group published the results of a meta-analysis on the role of EN for maintenance of remission in CD[80]. The main outcome measure was the occurrence of a clinical or endoscopic relapse. Two studies met the inclusion criteria and were included in the review. In the first, elemental and polymeric feeds (providing 35%-50% of the patients' calorie intake in addition to an unrestricted normal diet) were equally effective for maintenance of remission, allowing withdrawal of steroid therapy (OR 0.97, 95% CI: 0.24-3.92)[81]. In the second study, 51 patients with CD in remission were randomized to receive half their calories in the form of an elemental formula or to an unrestricted diet for up to 2 years[82]. The treatment group had a much lower relapse rate (34%) than the unrestricted diet group (64%), OR 0.3, 95% CI: 0.09-0.94). This study was halted before the expected end as a result of the interim analyses by the monitoring board, who found a significant benefit for the use of EN formula to maintain remission. Thus, the available evidence suggests that supplementary EN may be effective for maintenance of remission in CD.

Yamamoto investigated the impact of EN on the clinical and endoscopic recurrence after surgical resection for CD. Forty consecutive patients who underwent resection for ileal or ileocolonic CD were randomized to receive partial EN (EN group), or a regular diet (non-EN group). Ileocolonoscopy was performed at 6 and 12 mo after operation. Six months after operation, five patients (25%) in the EN group and 8 (40%) in the non-EN group developed endoscopic recurrence ($P = 0.50$). Twelve months after operation, endoscopic recurrence was observed in six patients (30%) in the EN group and 14 (70%) in the non-EN group ($P = 0.027$). One patient (5%) in the EN group and 7 (35%) in the non-EN group developed clinical recurrence during the 1-year follow-up ($P = 0.048$). Thus, long-term EN supplementation may significantly reduce clinical and endoscopic recurrence after resection for CD[65].

EXCLUSIVE ENTERAL NUTRITION (EEN) FOR INDUCTION OF REMISSION IN CHILDREN WITH CD

Pediatric studies showed that treatment with EEN can induce remission in up to 85% of newly diagnosed patients. The first meta-analysis of pediatric studies included five trials (147 patients). EEN was found as effective as corticosteroids in inducing remission (RR 0.95, 95% CI: 0.67-1.34)[83]. In a second meta-analysis in children, 11 RCTs ($n = 394$) were included[84]. Seven RCTs ($n = 204$) compared EEN with corticosteroid therapy. Based on the pooled results of four RCTs ($n = 144$), no significant differences in the remission rates between EEN and steroids were found (RR 0.97, 95% CI: 0.7-1.4). Four RCTs ($n = 190$) compared two EEN regimens[84]. Because of a lack of data, formal pooling of results was not possible for many outcomes (e.g. time until remission, duration of remission, growth data).

In conclusion, there are no differences in efficacy between EEN and corticosteroid therapy in the treatment of acute CD in children. Improved growth and nutritional status while avoiding the side effects of steroids make EEN the preferred choice for first-line therapy in children with active CD.

Johnson *et al*[85] investigated whether partial enteral nutrition (PEN) may be as effective as EEN in induction of remission in children with CD. They randomized children with active CD to either receive all of their nutrition as elemental diet (EEN) or only 50% (PEN). Total nutritional intake was similar in both groups, but the remission rate was higher in the EEN group (42%) then in the PEN group (15%)[85].

Elemental and polymeric formulae were shown to be equally effective in children with CD. In a randomized, non-blinded, multicenter, controlled trial in Sweden, 16 children with CD received Elemental 028 Extra (E028E) and 17 received Nutrison Standard (NuS). No significant difference was found between the 2 groups in remission rate at 6 wk (intent-to-treat analysis): E028E 11/16 (69%) and NuS 14/17 (82%), nor in the decrease in the Pediatric Crohn's Disease Activity Index (PCDAI) and adult CDAI. Patients treated with NuS gained more weight than patients with E028E. A polymeric diet may be superior to elemental diet in the treatment of pediatric CD where the primary aim is to increase the patient's weight[86].

EEN and disease location in children

Studies in children showed contradictory results with regard to the effect of disease location on EEN. Two studies found no difference in the remission rate of children with ileal *versus* isolated colonic disease[87,88]. In contrast, a recent report noted a decreased response rate in patients with isolated colonic disease[89]. EEN is beneficial in children with peri-anal disease, whether isolated or in combination with luminal disease[88]. As in adults, there is no convincing evidence that the effect of EN is restricted to small bowel disease only. The influence of disease location and other factors on response to EEN requires further evaluation.

Duration of remission/time to the first relapse in children

The meta-analysis of Dziechciarz *et al*[84] identified two RCTs ($n = 43$) that investigated duration of remission after EN. One study showed a significant reduction in

the time to relapse in the EN group compared with the corticosteroid group (n = 19, mean difference -0.4 year, 95% CI: -0.6 to -0.2)[22]. In the second RCT (n = 24), a similar trend was reported (the mean duration of remission was 7 mo in the EN group *versus* 10 mo in those treated with corticosteroids)[40].

The relapse rate after EN treatment is 50%-90% at 12 mo in adult studies. This is difficult to assess in the pediatric literature, as many of the reports are of selected groups with a short follow-up period, often of < 12 mo. Fell reported a follow-up of over 10 mo in 23 children who entered remission with EN, with 9 (39%) children relapsing (1 within 2 mo)[62]. The risk factors for early relapse are not well defined but probably include disease severity at onset, disease extent, and disease site, with colitis being more likely to relapse. There have been no long-term pediatric outcome studies after initial treatment with EN.

EN FOR MAINTENANCE OF REMISSION IN PEDIATRIC CD

Prospective studies investigating the role of long-term supplementation of a normal diet with EN in children with CD are limited[90,91]. Belli *et al*[92] reported on eight children with CD and severe growth failure whose clinical course was good on a regimen of elemental diet in one out of every 4 mo. Using this strategy they succeeded in reversing growth arrest, while decreasing prednisone requirements and PCDAI prior to puberty. Thus, maintenance EN can assist in maintaining remission, aid in ensuring adequate and appropriate growth, in addition to postponing the need for steroids.

LONG TERM OUTCOMES OF CHILDREN TREATED WITH EEN

Knight *et al*[88] retrospectively reviewed the long-term outcomes of 44 children treated with EEN over several years in a single pediatric IBD centre, with follow-up periods ranging between 1 and 7 years. Most children who continued maintenance EN had no relapse after remission was established and almost half of the patients have had no need for corticosteroids since diagnosis. In those who did require steroids, therapy was delayed by a median period of 68 wk (range 6-190). In addition, the authors showed improved weight Z scores 12 mo after diagnosis (compared to baseline values), but no improvement in height Z scores[88]. An additional pediatric retrospective study looked at 37 children who received EEN, comparing outcomes in these children to those of 10 children treated with steroids[93]. The initial remission rate in those managed with EEN was similar to that of corticosteroids (86.8% and 90%, respectively). Children managed with EEN achieved greater mucosal healing (64.8% *vs* 40%). Furthermore, EEN therapy led to significantly enhanced nutritional improvements and linear growth recovery compared to steroids. Both groups were managed with maintenance amino salicylic acid (5-ASA) therapy. The EEN treated group had a much longer duration of remission in the 12-mo follow-up period[93].

QUALITY OF LIFE AND EEN

Administration of EEN has been found to be difficult in adult patients as reflected by treatment dropout rates as high as 55%. The influence of therapy on quality of life (QOL) during and following EEN were evaluated in two recent studies of children with CD. One showed improved QOL scores in 24 of 26 children treated with EEN for active CD, with 90% of the children achieving remission. Even the three children who received their enteral formula *via* a nasogastric tube reported overall improvements in QOL[94]. Children receiving EEN through nasogastric tubes emphasized the difficulties associated with the use of these tubes. However, it seems that the improvements in bowel symptoms and overall well-being may outweigh the negative aspects[95].

SIDE EFFECTS OF EEN

EEN is safe and generally well tolerated. Side effects are minimal (23.5%) and include nausea, abdominal pain, flatulence, or diarrhea[96]. The only reported severe adverse event associated with EEN is a single case of refeeding syndrome[97].

DIETARY FACTORS AND THE RISK OF IBD

Several studies examined the association between specific dietary patterns and the risk of CD, including the amount of energy, fat type and quantity, carbohydrates, specific amino acids and fiber. This issue is beyond the scope of this review and the reader is referred to previous and recent publication on dietary factors predisposing to IBD in adults and children[98-100].

CONCLUSION

Malnutrition is common in IBD, the etiology is multifactorial, and it is associated with adverse consequences. Its management requires identification and treatment of the nutritional deficits. PN may correct nutritional deficits and maintain nutritional status. However, EN has similar efficacy to PN with lower costs and fewer complications and is thus the modality of choice. Several studies have attested the efficacy of enteral formulations to control disease activity in adult CD patients. EN is considered the therapy of choice for children with active CD especially in the presence of growth retardation. It was shown to induce clinical remission, mucosal healing, modulate mucosal immune events, nutritional improvement and resumption of growth.

Therefore, in children, EN should be considered as the treatment of choice when acceptable by the family and child. Although not efficacious as steroids in

inducing remission in adults with CD, nutritional therapy has the advantages of controlling inflammation, mucosal healing and overall nutritional status with minimal adverse effects.

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REVIEW

Renin-angiotensin system in the pathogenesis of liver fibrosis

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Abstract

Hepatic fibrosis is considered a common response to many chronic hepatic injuries. It is a multifunctional process that involves several cell types, cytokines, chemokines and growth factors leading to a disruption of homeostatic mechanisms that maintain the liver ecosystem. In spite of many studies regarding the development of fibrosis, the understanding of the pathogenesis remains obscure. The hepatic tissue remodeling process is highly complex, resulting from the balance between collagen degradation and synthesis. Among the many mediators that take part in this process, the components of the Renin angiotensin system (RAS) have progressively assumed an important role. Angiotensin (Ang) Ⅱ acts as a profibrotic mediator and Ang-(1-7), the newly recognized RAS component, appears to exert a counter-regulatory role in liver tissue. We briefly review the liver fibrosis process and current aspects of the RAS. This review also aims to discuss some experimental evidence regarding the participation of RAS mediators in the pathogenesis of liver fibrosis, focusing on the putative role of the ACE2-Ang-(1-7)-Mas receptor axis.

Key words: Hepatic fibrosis; Renin angiotensin system; Angiotensin Ⅱ; Angiotensin-(1-7); Receptor Mas; Angiotensin converting enzyme 2

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INTRODUCTION

Liver fibrosis is a dynamic process resulting in excessive deposition of extracellular matrix (ECM) components. It is a multifunctional process that involves hepatic stellate cell (HSC) and Kupffer cells (KCs), cytokines, chemokines and growth factors and results from a disruption of homeostatic mechanisms that maintain the liver ecosystem[1-3]. The fibrogenic cascade can be divided into the following steps[1-3]: (1) activation of HSC and KCs; (2) migration and proliferation of HSCs; (3) synthesis and deposition of ECM components; (4) remodeling of scar tissue; (5) wound contraction; (6) apoptosis of HSCs.

In this manuscript, we briefly review the liver fibrosis process and current aspects of the Renin angiotensin system (RAS) and further discuss the putative role of Angiotensin (Ang)-(1-7) in controlling hepatic injury.

LIVER FIBROSIS PROCESS

The HSC is the main cell type responsible for excessive deposition of connective tissue components, including

type I collagen, in response to liver injury[1-6]. HSCs, also called lipid storage cells, lipocytes or Ito's cells, are found in the space of Disse among endothelial cells and hepatocytes. These cells represent approximately one third of the non-parenchymatous cell population or 15% of the total number of hepatic cells[2-4]. The main function of HSCs is to metabolize vitamin A, which is intracellularly reserved as cytoplasmatic fat bodies, mainly as retinol esters[4]. Such cells also contain a small amount of triglycerides, phospholipids, cholesterol and free fatty acids. Moreover, they produce cytokines, growth factors and inflammatory mediators[4-6]. The activation of HSCs is a process not fully understood that involves the depletion of vitamin A storage and the lowering of retinol chains[2,4]. There are also important morphological and functional changes in the activated HSC, which include the increase in the expression of myogenic and neurogenic proteins and the subsequent change into highly contractile fibroblasts[2-4]. HSCs, despite being firmly adhered to hepatic endothelial cells, also play a pivotal role in the regulation of portal pressure. Besides that, due to the expression of multiple actin and muscular and non-muscular myosin types, when the HSC transforms itself into a myofibroblast, it acquires the ability to contract scar tissue and fibrous septa[1-3].

Formal pathogenesis of fibrosis is initiated by parenchymal cell destruction (necrosis rather than apoptosis) due to multiple injurious agents and mechanisms followed by inflammation, which in turn activates "resting" HSCs[1-3]. The HSCs lose their lipid droplets, proliferate, migrate to the third zone of the hepatic lobule, modify themselves in order to acquire a myofibroblast-like phenotype and start producing collagen types Ⅰ, Ⅲ and Ⅳ and laminin[2-4]. It is generally accepted that α actin expression reflects the activation of HSC[4]. Activated HSCs migrate to injured sites and proliferate in response to numerous pro-fibrogenic mediators, among which transforming growth factor β-1 (TGFβ-1) and platelet-derived growth factor are considered the most effective ones[7,8]. HSCs are the main cells responsible for ECM production in the liver. The ECM serves as a storage site for cytokines and growth factors, and thus, tissue injury could induce their release[2,4]. This event may be responsible for providing the initial signal for tissue repair, prior to the activation of cells within the liver and/or the arrival of inflammatory cells (Figure 1).

Activated HSCs express and secrete matrix molecules, cytokines and chemokines, matrix metalloproteinases (MMPs) and their respective tissue inhibitors of metalloproteinases (TIMPs)[1-4]. Thus, HSCs participate pathophysiologically both in fibrogenesis and fibrolysis, i.e. enzymatic dissolution of the ECM and, thus, in tissue remodeling. According to this hypothesis, fibrosis is conditionally reversible, based on the fact that HSCs produce multiple MMPs, which degrade interstitial and basement membrane collagens[9-11]. Thus, when the fibrogenic stimulus is singular, or multiple stimuli have not induced a state in which the excess

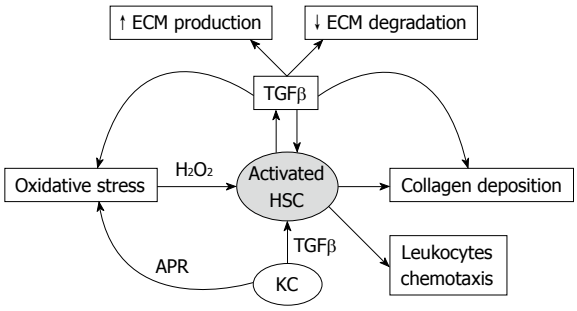

Figure 1 Diagram of activated hepatic stellate cell actions and interactions in liver fibrosis process. HSC: Hepatic stellate cell; KC: Kupffer cell; APR: Acute phase response; ECM: Extracellular matrix; TGFβ: Transforming growth factor-β.

deposition of ECM components is accompanied by a distortion of liver architecture, the process may be completely reversible[2,5,9-11]. At this stage of liver fibrosis, interruption of the fibrogenic stimulus results in MMPs secretion[11]. However, when the fibrogenic process is already associated with formation of connective tissue septa, distortion of liver architecture and formation of vascular shunts, fibrosis becomes irreversible, unless one finds the means to stimulate production and activation of MMPs, to down-regulate the expression of TIMPs, and to inhibit the production of collagen[2,5,9-13].

Another cell line involved in liver fibrogenic cascade is the KC. KCs are highly mobile macrophages that are attached to the endothelium[1-3]. Physiologically, KCs produce the immunosuppressive cytokine, interleukin (IL)-10, that prevent HSC proliferation and/or collagen synthesis[1,2,4]. These cells are activated by engulfment of apoptotic hepatocytes; this leads to removal of dead cells from the liver. Furthermore, activated KCs secrete inflammatory cytokines, linking apoptosis in the liver to inflammation. Once there is hepatic injury, activated KCs possess autocrine and paracrine loops that induce the expression of TGFβ, which, in turn, promotes HSC and hepatocyte proliferation and/or chemotaxis of inflammatory cells and HSCs[1,2,4,14,15]. In these conditions, circulating levels of cytokines and chemokines, such as tumor necrosis factor-α (TNFα), IL-6, IL-1, IL-8, colony stimulating factor, monocyte chemotactic protein and leukotrienes, are increased. These elevated mediators have a key role in recruiting neutrophils and monocytes into the lesion site and exert an important regulatory effect on the expression of collagen genes by HSCs[1-3]. Indeed, KCs have a pivotal role in the activation of HSCs and/or in increasing their capacity to produce ECM components during hepatic diseases[1-3].

Among all cytokines and growth factors, the TGFβ superfamily exerts important functions during the development, differentiation and tissue remodeling[14,15]. TGFβ1 strongly modulates cell proliferation by increasing ECM proteins and proteases inhibitors and by diminishing several metalloproteases expression[1-3,12-15]. In normal hepatic tissue, TGFβ-1 and TGFβ-2 mRNA are predominantly expressed by KCs, while TGFβ-3 is detected in stellate cells. During fibrogenesis, TGFβ-2

and TGFβ-3 expression are diminished, while TGFβ-1 expression is significantly increased among stellate and endothelial cells[1-3,12-15]. This cytokine is a potent inhibitor of hepatocyte proliferation, but it is also capable of regulating hepatocyte growth during its regeneration. TGFβ induces the formation of oxygen reactive species, which is involved in HSC activation and in the augmentation of mRNA expression for collagen I [1-3,12-15].

Depending on the magnitude of the injury, the host's response may be local and/or systemic. When the events take place in the liver, the response is restricted to HSCs and KC activation or results in recruiting inflammatory cells that, along with KCs, produce cytokines and growth factors necessary to the healing process[2,3,12-15]. When the injury takes a larger extent and local events cannot control it, there is a systemic response, common to every other inflammatory process, regardless of the causal agent. This systemic reaction corresponds to the acute phase response, characterized by the increased production of TNFα, IL-6, IL-1, oncostatin M and acute phase proteins[1-3]. Although these changes are intended to limit the tissue injury, elevated cytokine expression, mostly IL-6, may contribute to hepatic fibrosis by enhancing ECM deposition, collagen I content and fibronectin genetic transcription, as well as by stimulating other fibrogenic cytokines (such as TGFβ), and by amplifying TIMP production[1-3,11-15].

There are other factors involved in the ECM remodeling process, which include MMPs and TIMPs. MMPs 1 and 13, also known as collagenases 1 and 3, respectively, are the main secreted neutral proteinases capable of initiating degradation of collagen types Ⅰ, Ⅲ and Ⅴ[11]. The individual contribution of MMPs in ECM degradation within the normal liver and during hepatic fibrogenesis remains unclear. HSCs express the MMP 1 mRNA gene, but the enzyme levels are not increased in patients with fibrosis. On the other hand, MMP 13 expression is augmented at early phases of liver fibrosis, preceding the increase of collagen I production[12,13]. Among other MMPs, types 2 and 3 have their expression elevated in HSCs during intermediate and initial phases, respectively, of carbon tetrachloride induced hepatic fibrosis[3,10,12,13]. MMP 9, also called gelatinase B, can degrade collagen IV, gelatin and laminin, thus facilitating cellular migration through basement membranes[3,12,13]. There are also TIMPs that regulate enzyme activity and play a role in different models of hepatic fibrosis[5]. Summing up, the hepatic tissue remodeling process is highly complex, resulting from the balance between collagen degradation and synthesis (Figure 1).

The understanding of the HSC activation pathways and the approach of molecular biology have provided new strategies to hepatic antifibrotic therapy[1-4]. Many of these strategies are based on the inhibition of collagen deposition and/or inactivation of the HSCs. Experimental studies in which the treatment is provided simultaneously and/or during the course of fibrosis induction have been successful[1,2,9-11]. Such approaches may include: (1) healing the primary disease in order to prevent the injury[1,2]; (2) reducing inflammation or the host's response to avoid HSC activation (interferon-α, ursodesoxycholic acid, corticosteroids and TNF-α antagonists)[1,2,9]; (3) direct inhibition of HSC activation (antioxidants-vitamin E and interferon γ-endothelin receptor antagonists)[1,2,9]; (4) neutralizing the HSCs proliferative, fibrogenic, contractile and/or pro-inflammatory response [angiotensin converting enzyme (ACE) inhibitors, angiotensin Ⅱ type 1 receptor (AT_1) antagonists, Ang-(1-7) receptor Mas agonists, proteases inhibitors, hepatocyte growth factor, tyrosine kinase inhibitors, endothelin receptor antagonists][9-11]; (5) stimulating HSC apoptosis (gliotoxin, Fas ligands)[9-11]; (6) increasing scar matrix degradation by stimulating proteases producer cells and by providing such proteases (TGFβ antagonist, MMP tissue inhibitors)[9-11].

Nevertheless, liver fibrosis in humans is a silent disease. Many patients are diagnosed in an advanced phase when fibrous septa and hepatic architecture distortion already exist. Thus, the development of new treatments focusing on the removal of fibrous septa and promoting hepatic tissue regeneration becomes essential.

CURRENT ASPECTS ON THE RAS

The RAS is classically conceived as a hormonal cascade responsible for controlling cardiovascular, renal and adrenal functions that regulate hydro-electrolytic balance and blood pressure through Ang Ⅱ actions[16].

Recent advances in cellular and molecular biology, as well as physiological and pharmacological approaches, have generated new concepts through the identification of new peptides, enzymes, receptors and biological actions. Additionally, tissue RAS has been characterized in different organs and systems, in which significant interactions between receptors, mediators and metabolic pathways have been discovered[17-21]. In this field, some of the latest advances should be mentioned: (1) the characterization of other biologically active RAS fragments, besides Ang Ⅱ, such as Ang Ⅲ, Ang Ⅳ and Ang-(1-7)[17-21]; (2) the discovery of a new enzyme, a homolog to the ACE, called ACE2[22,23], which is the main enzyme responsible for the conversion of Ang Ⅱ into Ang-(1-7)[24,25]; (3) the identification of the G protein-coupled receptor Mas, a functional receptor for Ang-(1-7)[26]. These discoveries have contributed to the understanding of RAS in normal physiology and in pathological conditions[19-21].

Among RAS mediators, Ang-(1-7) is of particular interest due to its selectivity, which is attributed to the absence of phenylalanine (Phe) in the C-terminal position, which is critical for the binding of Ang Ⅱ to AT_1 receptors[27]. Several enzymatic routes may be involved in Ang-(1-7) formation, either directly from Ang I or from Ang Ⅱ through tissue peptidase actions, including neutral endopeptidase, oligopeptidase, prolyl-carboxypeptidase, prolyl-endopeptidase and ACE2[24,28]. The recent discovery of ACE2 has provided

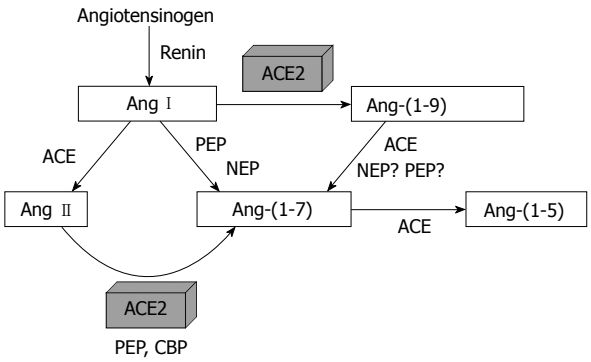

Figure 2 View of the main metabolic pathways of Ang Ⅱ and Ang-(1-7). ACE: Angiotensin converting enzyme; ACE2: Angiotensin converting enzyme 2; PEP: Prolyl-endopeptidase; NEP: Neutral endopeptidase; CBP: Carboxypeptidase.

an important pathway for production of Ang-(1-7), probably by exerting an important role in tissue peptide formation[19-21]. Figure 2 shows the main pathways for Ang-(1-7) metabolism.

Once it is formed, Ang-(1-7) is rapidly hydrolyzed, especially by ACE[29]. In the presence of ACE inhibition and after chronic administration of AT_1 receptor blockers, Ang-(1-7) levels are raised several times, suggesting that this heptapeptide contributes to the actions of RAS blockers[28,30,31]. Many studies have indicated the physiopathological importance of Ang-(1-7) in human diseases[32-36]. In general, Ang-(1-7) has opposing actions to Ang Ⅱ, which gives the system a dual influence over various tissues[20,21]. For instance, Ang-(1-7) have vasodilator and anti-proliferative effects on blood vessels[37-39]. This effect allows RAS to influence vascular growth, either by stimulating through Ang Ⅱ or inhibiting through Ang-(1-7)[21,37-39]. Ang Ⅱ is clearly mitogenic in vascular smooth muscle cell (VSMC) culture and in intact arteries, whereas Ang-(1-7) inhibits VSMC growth and reduces neointimal formation[37,38,40]. To date, Ang Ⅱ and Ang-(1-7) exhibit opposite effects on the regulation of cell growth as demonstrated by Gallagher and Tallant (2004) in lung cancer cells[41]. Receptor Mas appears to be involved in the antiproliferative effect of Ang-(1-7) in VSMC[42] and in stent-induced neointima proliferation[43]. Furthermore, it has been demonstrated that Ang-(1-7) inhibits vascular growth through prostaglandin-mediated intracellular events inducing cAMP production and reduction of Ang Ⅱ-stimulated ERK1/2 activities[44].

RAS can be envisioned as a dual function system, in which the vasoconstrictor/proliferative or vasodilator/antiproliferative actions are primarily driven by the ACE/ACE2 balance[21,45]. According to that, an increased ACE/ACE2 activity ratio will lead to increased Ang Ⅱ generation and increased catabolism of Ang-(1-7) favoring vasoconstriction while an opposite ratio will decrease Ang Ⅱ and increase Ang-(1-7) levels facilitating vasodilation[21,45]. The fact that Ang-(1-7)/Mas directly antagonizes many actions of Ang Ⅱ, provides an additional layer of counter regulation in the system[46].

THE RAS'S ROLE IN LIVER FIBROSIS

A growing body of evidence indicates that the RAS takes part in the pathogenesis of liver fibrosis[47-61]. In this regard, plasma renin activity and aldosterone levels were both elevated in patients with liver cirrhosis, especially those with hepatorenal syndrome[47-50]. Indeed, Ang Ⅱ and aldosterone actions lead to renal vasoconstriction, blood flow redistribution and increases in sodium and water tubular reabsorption[47-49]. All of these effects tend to normalize the organ perfusion pressure and plasma effective volume. The elevation in circulating levels of PRA and aldosterone is due to an excessive production of these substances and not from a diminished hepatic catabolism[47,48]. This higher production could be attributed either to a physiological response to systemic vasodilation that occurs in cirrhotic patients[48,61] or to an intrahepatic RAS activation[53-60].

Tissue fibrosis is a common response in numerous chronic diseases, regardless of etiology, resulting in the production of, for example, liver cirrhosis, glomerulosclerosis, interstitial lung fibrosis and cardiac hypertrophy. Thus, resembling what happens in renal[62,63] and cardiac[16] fibrosis, several studies suggest that Ang Ⅱ could mediate and exacerbate liver fibrosis through HSC activation and by stimulating TGFβ-1 *via* AT_1 receptors[1-6,14,15,53-55]. Bataller *et al*[53] have shown the presence of AT_1 receptors in human activated HSC cultures. Experimental studies with AT_{1a} receptor knockout mice showed an attenuated liver inflammation and fibrosis following bile duct ligation[56]. Immunohistochemistry analysis revealed decreased infiltration by inflammatory cells, reduced lipid peroxidation products and decreased phosphorylation of c-Jun and p42/44 MAPK in AT_{1a} knockout mice compared to AT_1 wild type animals[56]. On the other hand, the genetic deletion of Ang Ⅱ AT_2 receptors worsened the fibrosis induced by CCl_4 by stimulating oxidative stress, which lead to HSC activation[57]. While AT_1 receptors play an important role in the development of fibrosis, the AT_2 signal has anti-fibrogenic and/or cytoprotective effects on oxidative stress-induced liver fibrosis[56,57]. Taken together, these experimental studies suggest that RAS-associated liver fibrogenesis may be determined by the balance between AT_1 and AT_2 signals.

Therefore, by activating AT_1 receptors, Ang Ⅱ induces contraction and proliferation of HSCs, which is considered the principal effector of hepatic acinar fibrosis[53-55]. A similar effect has been observed in mesangial and VSMC. AT_1 receptors are found in most of the mesenchymal cells and mediate the majority of the Ang Ⅱ biological effects, including the increase in intracellular calcium, cellular contraction and proliferation[58,59]. The magnitude of the HSC contractile response to Ang Ⅱ is comparable to the effect elicited by endothelin-1, which is considered the most powerful contractile agent to this cell line. Ang Ⅱ contractile effects were attenuated in the presence of powerful vasodilators, such as nitric oxide and prostaglandins[53], and completely blocked by pre-incubation with the AT_1 receptor blocker, losartan[55]. Additionally, Ang Ⅱ

mediates key biological actions involved in hepatic tissue repair, including myofibroblast proliferation, infiltration of inflammatory cells, and collagen synthesis[60]. Activated HSCs secrete Ang Ⅱ, which induces fibrogenic actions through the activation of NADPH oxidase[1-4].

Although the mechanisms of hepatic fibrosis are not fully understood, such as in other tissues, experimental evidence indicates that TGFβ-1 has a key role in this process[14,15]. In the heart and in the kidneys, many vasoactive peptides have shown themselves capable of enhancing TGFβ-1 expression, including Ang Ⅱ[16,60,62]. Jonsson *et al*[54] have investigated functional polymorphisms of TGFβ-1 and angiotensinogen genes and the influence of these genotypes in liver fibrosis of patients with chronic C hepatitis. These authors found a significant relation between TGFβ-1 and angiotensinogen genotypes and the development of liver fibrosis[54]. Patients that did not exhibit a profibrotic genotype normally did not develop fibrosis. Ang Ⅱ also increases TGFβ-1 and the genetic expression of collagen 1 *via* AT_1 receptors in the liver[55,58]. TGFβ-1 also induces HSC activation, which, in turn, increases TGFβ-1 expression[14,15]. Thus, there is a formation of autocrine and paracrine loops that assure the continuous production of this fibrogenic cytokine[1,6,14,15].

RAS inhibition reduces collagen Ⅳ expression and interstitial expansion in different tissues. The response to treatments with AT_1 receptor blockers and ACE inhibitors clearly illustrate the importance of the RAS in renal and cardiac fibrosis[16,17,64,65]. Kidney tubulointerstitial fibrosis induced by cyclosporine was ameliorated by RAS inhibition[66]. Similarly, RAS pharmacological blockade also reduced collagen Ⅳ expression and interstitial expansion in rats with renal obstruction[67]. Treatment with ACE inhibitors and/or AT_1 receptor blockers has also shown beneficial effects in liver diseases[51,52,54,55,58]. Some studies have shown reductions in TGFβ-1 and procollagen α1 mRNA levels in the liver of rats treated with captopril after common bile duct ligation (BDL), supporting the hypothesis of an Ang Ⅱ action on HSCs[58,59]. In addition to antifibrotic effects, captopril has improved hemodynamic alterations, renal function and cholestasis[51,52,54,55,58]. Paizis *et al*[59] demonstrated that the RAS blockade by irbesartan, an AT_1 receptor antagonist, in BDL rats, reduced the expression of TGFβ-1 and of collagen 1 in the liver. These findings are consistent with the concept that the Ang Ⅱ-TGFβ-1 axis may work in the liver as a pathway towards organic fibrosis, as previously demonstrated in other experimental models, like the use of carbon tetrachloride[68] in mansonic schistosomiasis[69] and, more recently, in transgenic mice with high expression of TGFβ-1[70]. In fact, the local production of Ang Ⅱ, as well as circulating RAS activation, may be a significant part of the tissue overall response to injuries.

However, there are few studies exploring the role of Ang-(1-7) in liver fibrosis[45,71-75]. Our group recently showed that the progression of liver dysfunction in BDL rats is characterized by marked changes in Ang-(1-7) levels and that the overall activation of the circulating

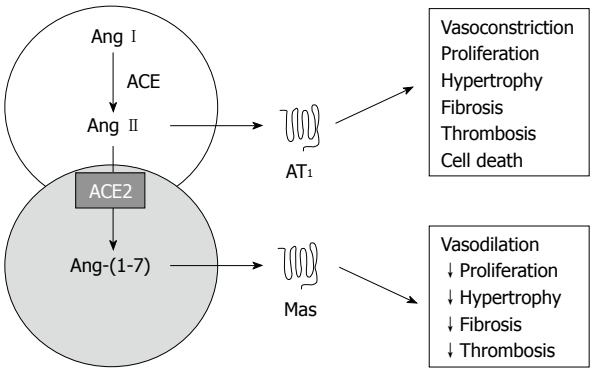

Figure 3 A schematic diagram of both RAS arms. The counter-regulatory arm of the RAS, ACE2-Ang-(1-7)-Mas axis, produces effects that oppose those of the ACE-Ang Ⅱ-AT_1 receptor axis. Ang: Angiotensin; Mas: G-protein coupled receptor of Ang-(1-7); AT_1: Type 1 receptor of Ang Ⅱ.

RAS was associated in time with the progression of hepatic fibrosis[72]. Furthermore, the pharmacological blockade of the Ang-(1-7) receptor Mas accelerated liver fibrosis by increasing the liver content of collagen and TGFβ-1[72]. In line with these findings, Paizis *et al*[71] (2005) observed an upregulation of ACE2 and its widespread expression throughout the liver in BDL animals and in human cirrhosis. A few years later, the same group showed that as BDL rats developed advanced fibrosis, increased expression of components of the classic RAS such as ACE, AT_1 receptor and Ang Ⅱ was accompanied by increased hepatic and plasma ACE2 activity, increased Mas expression in the liver and a major rise in plasma levels of Ang-(1-7)[73]. More recently, Lubel *et al*[75] (2009) corroborated these previous studies regarding the protective role of Ang-(1-7) against liver fibrosis. The authors reported that, in BDL rats, Ang-(1-7) not only improved the histological fibrosis stage and reduced hydroxyproline content but also decreased gene expression of collagen 1A1, α-SMA, VEGF, CTGF, ACE and receptor Mas[75]. In addition, cultured hepatic cells expressed AT_1 and Mas receptors, and when treated with Ang-(1-7) or the Mas receptor agonist, AVE 0991, produced less α-SMA and hydroxyproline, an effect reversed by the Mas receptor antagonist, A779. Ang-(1-7) is upregulated in human liver disease and has antifibrotic actions in a rat model of cirrhosis[75]. Indeed, the current studies[45,71-75] raise the possibility that upregulation of hepatic ACE2 and Mas, and the generation of Ang-(1-7) represent a counter regulatory response to RAS-mediated liver injury (Figure 3).

There is substantial evidence to suggest that Ang-(1-7) is involved in the beneficial actions of AT_1 receptor blockers, ACE and vasopeptidase inhibitors[76]. Supporting this theory Maia *et al*[77] have shown that the antagonist of Ang-(1-7) receptor Mas, A-779[78], has attenuated the hypotensive response to bradykinin in animals treated with ACE inhibitors, suggesting the involvement of Ang-(1-7) in the cardiovascular effects of ACE inhibitors. The studies with BDL rats are also evidence that RAS blocking agents may attenuate liver

fibrosis not only by antagonizing Ang Ⅱ, but also by elevating Ang-(1-7) levels[45,72-75]. Thus, the administration of Ang-(1-7) or its oral agonist, the compound AVE0991[75,79], could be useful for understanding the mechanisms of fibrosis and should be further investigated for the treatment of liver diseases associated with fibrosis.

CONCLUSION

The better understanding of the underlying mechanisms involved in liver fibrosis makes effective antifibrotic therapy an imminent reality. However, treating this disease remains a challenge and, up to this moment, no antifibrotic agent has been approved for routine human use. It is important to mention that Ang-(1-7) is quickly hydrolyzed, especially by ACE and, in presence of ACE inhibition and after chronic administration of AT_1 receptor blockers, its levels increase several times[30,31,35], suggesting that this heptapeptide may contribute to RAS blockade[20,21]. Furthermore, Kostenis *et al*[46] have recently demonstrated that the Ang-(1-7) Mas receptor can hetero-oligomerize with the AT_1 receptor and by so doing inhibits the actions of Ang Ⅱ. So, it is believed that the Mas receptor acts *in vivo* as an antagonist to the AT_1 receptor[46]. Hence, it has raised the hypothesis that the RAS acts through two pathways: the first one, responsible for the main actions of the system, composed of the ACE-Ang Ⅱ-AT_1 receptor system and the second one, the counter-regulatory pathway, formed by the ACE2-Ang-(1-7)-receptor Mas system[20,21,45]. Finally, the use of ACE inhibitors, AT_1 receptor antagonists and, perhaps, Ang-(1-7) receptor Mas agonists[78] could become important tools in this study, and maybe to the therapeutic approach of liver fibrosis.

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Prognostic factors and time-related changes influence results of colorectal liver metastases surgical treatment: A single-center analysis

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Abstract

AIM: To analyze the prognostic factors involved in survival and cancer recurrence in patients undergoing surgical treatment for colorectal liver metastases (CLM) and to describe the effects of time-related changes on survival and recurrence in these patients.

METHODS: From January 1994 to January 2006, 236 patients with CLM underwent surgery with the aim of performing curative resection of neoplastic disease at our institution and 189 (80%) of these patients underwent resection of CLM with curative intention. Preoperative, intraoperative and postoperative data, including primary tumor and CLM pathology results, were retrospectively reviewed. Patients were divided into two time periods: a first period from January 1994 to January 2000 (n = 93), and a second period from February 2000 to January 2006 (n = 143).

RESULTS: Global survival at 1, 3 and 5 years in patients undergoing hepatic resection was 91%, 54% and 47%, respectively. Patients with preoperative extrahepatic disease, carcinoembryonic antigen (CEA) levels over 20 ng/dL, more than four nodules or extrahepatic invasion at pathological analysis had worse survival. Tumor recurrence rate at 1 year was 48.3%, being more frequent in patients with preoperative and pathological extrahepatic disease and CEA levels over 20 ng/dL. Although patients in the second time period had more adverse prognostic factors, no differences in overall survival and recurrence were observed between the two periods.

CONCLUSION: Despite advances in surgical technique and better adjuvant treatments and preoperative imaging, careful patient staging and selection is crucial to continue offering a chance of cure to patients with CLM.

Key words: Liver metastases; Colorectal cancer; Hepatic resection; Survival; Prognostic factors

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INTRODUCTION

Up to 50% of patients with colorectal carcinoma (CRC) will develop metastases during the course of their disease, leading to certain death if untreated[1,2].

Colorectal liver metastases (CLM) are present in 15% to 25% of cases at the time of diagnosis of primary tumor, and another 25% to 50% will develop metachronic CLM within 3 years following resection of primary CRC[1,3-6].

Surgical resection remains at the present time the only potentially curative treatment for patients with CLM, even though hepatic resection is only possible in less than 25% of patients with metastatic disease limited to the liver[1,5,6]. Although neoplastic recurrence is observed in up to 50% of patients and remains a basic determinant of survival, only 20%-30% of these patients are potentially amenable to repeat hepatic resection[7,8]. Five-year survival after curative resection ranges 30%-40%, whereas less than 2% of patients are alive 5 years after diagnosis without surgical therapy[5,6,9].

Many attempts have been made to classify patients into stratification groups in order to determine which patients would obtain most benefit from resection, even though the most used classification remains the clinical risk score (CRS) described by Fong *et al*[9-11]. According to these scores, CLM resection should be put into question in high-risk patients because of poor expected results after surgery, and therefore these patients should be included in chemotherapeutical trials. In recent years an increase in indications for resection of CLM has been observed due to a multidisciplinary approach with improvements in surgical techniques, anesthetic management and the introduction of new chemotherapy drugs[12-15]. This approach has led to an extension of the traditional limits of CLM resectability with promising results, although the number of patients is still too small to draw definitive conclusions[16,17].

Taking all these facts into consideration, the aims of our study were to analyze the survival and recurrence of patients with CLM undergoing surgical treatment in our hospital, to determine whether any single factor was significantly associated with survival and recurrence, and to describe the effects of time-related changes in our series of patients.

MATERIALS AND METHODS

Between January 1994 to January 2006, 236 consecutively recruited patients with CLM were operated on in our institution with the aim of performing curative resection of neoplastic disease. Among them, 189 patients (80%) underwent curative hepatic resection (defined as resection of all macroscopic neoplastic tissue found during laparotomy), with 18 patients undergoing resection of extrahepatic disease at the same time.

CLM resection criteria at the hospital clinic

Criteria for resection of CLM have changed over time and, in general, these indications were more restrictive at the beginning of the series and have expanded as surgical experience and better perioperative care of patients has been acquired. The classically accepted indications for CLM surgery in our institution are: (1) patients presenting with 4 or less nodules, (2) remaining liver parenchyma over 25% of total liver volume and (3) no extrahepatic neoplastic disease (defined as the presence of metastatic neoplastic tissue beyond the limits of hepatic capsule). However, the definitive guiding criteria used to consider or refuse a patient for CLM resection is the possibility of benefit from a complete resection of neoplastic disease with enough functional residual liver, despite the localization and number of lesions and an adequate physical status to tolerate liver resection (defined by a performance status under 3 and an absence of serious associated illnesses). All prospective surgery patients with CLM are evaluated and staged with radiological studies (chest X-ray, abdominopelvic CT or MRI scan, occasionally complemented with liver volumetry in cases when predicted liver remnant is small, as well as PET scan in cases when extrahepatic invasion is suspected), laboratory tests including liver function tests, carcinoembryonic antigen (CEA) assay and a complete colonoscopy. Candidates are presented to a CRC committee meeting composed of liver and colorectal surgeons, oncologists and radiologists.

Liver resection technique for CLM

Despite several changes in the resection technique during the time considered in the study, the main technical points for CLM resection have remained constant over time (J-shaped skin incision in the upper right quadrant, intraoperative ultrasonography (IOUS) and liver transection with an ultrasonic dissector under Pringle maneuver if needed).

Although the increasing use of laparoscopic techniques in colorectal resections has led to a reduction in the amount of surgical trauma[18], thus making one-step resection of CRC and CLM possible, the majority of our patients with synchronous CLM usually undergo colorectal and liver resection in two separate stages. In cases when simultaneous resection is planned, patients are operated on by two coordinated different teams of surgeons specialized in hepatic and colorectal surgery respectively. Colorectal resection is always performed first and the decision about performing liver resection at the same operation is taken depending on the type and extent of colorectal and liver resection, and any intraoperative findings that might recommend a two-stage procedure.

Postoperative follow-up of patients with CLM

After discharge from hospital, patients are followed by a multidisciplinary team of oncologists, colorectal and hepatic surgeons and postoperative status and pathologic results are then reviewed. Depending on the previous treatments, overall risk and tolerance, patients are proposed to be treated with complementary chemotherapy, mainly based on 5-fluorouracil and either irinotecan or oxaliplatin, although in recent times the use of cetuximab as an adjuvant agent has increased. Usual postoperative follow-up in order to detect neoplastic recurrence consists of physical examination, laboratory

tests with liver function tests and CEA assay, and abdominal ultrasonography or CT every 3 mo during the first 2 years and every 6 mo after the second year.

CLM resection data

Patients were classified according to the interval between the diagnosis of CLM and CRC resection. CLM diagnosed before, during or within 90 d of CRC resection were classified as synchronous and those CLM diagnosed at least 90 d after CRC resection were classified as metachronous.

In order to study the evolution of CLM resection over time, the entire series of patients was divided into two periods of equal length: the first period from January 1994 to January 2000 and the second period from February 2000 to January 2006.

Preoperative, intraoperative and postoperative data including CRC and CLM pathology results were retrospectively reviewed.

Definitions of interventions and results

Combined hepatectomy was defined as any major (three or more segments) or minor (less than three segments) hepatectomy with any associated atypical (non-anatomical) resection.

Neoplastic recurrence was diagnosed by at least two coinciding image techniques or surgical exploration at least 30 d after liver resection. Survival was calculated using the last follow-up date (January 31, 2008) or the date of expiration.

Statistical analysis

Categorical variables were compared using the chi-square or Fisher's exact test. Continuous variables were expressed as mean ± SD and compared using Student's *t* test. When a normal distribution was not present, continuous variables were expressed as the median and the range and compared using the Mann-Whitney *U* test.

Patient survival and recurrence were calculated using the method of Kaplan-Meier, and the log rank test was used to compare survival in the univariate analysis. Multivariate analysis was calculated using a Cox regression model.

A *P* value under 0.05 was considered significant. All statistical analyses were performed with the "Statistical Package for the Social Sciences" version 11.0 for Windows (SPSS, Chicago, IL).

RESULTS

Demographic and preoperative data of the patients in the series are shown on Table 1. Overall median follow-up was 5.8 years, with a minimum follow-up of 1 year and maximum of 14 years. By time periods, median follow-up in the first period group was 9.3 years, while in the second period group was 3.8 years.

Primary tumor-related and preoperative factors

Synchronous metastases, colonic localization of the primary tumor and parameters related to a low risk CRS accounted for the majority of patients in the series, even though some cases had extreme size and number of CLM. Over half of the patients had received some adjuvant treatment for CRC (five patients having received radiotherapy, 97 patients having received chemotherapy and 39 patients having received both treatments). Preoperative treatment before hepatic resection was given to 62 patients, the majority of them receiving 5-fluorouracil-based systemic chemotherapy because of synchronous or initially non-resectable metastases (Table 1).

Table 1 Demographic and preoperative characteristics of the patients *n* (%)

Characteristics	
Total patients	236
Age (yr) (mean, range)	63 (36-81)
Sex (male/female)	153/83
Metachronous metastases (> 3 mo)	88
Synchronous metastases	137
Previous CLM resection	11
Localization of primary tumor	
Rectum	71 (30)
Colon	165 (70)
Differentiation of primary tumor	
Poor differentiated	32 (14.4)
Moderately differentiated	176 (78.9)
Well differentiated	15 (6.7)
Adjuvant treatment of primary tumor	
Chemotherapy	97 (41.1)
Radiotherapy plus chemotherapy	39 (16.5)
Radiotherapy alone	5 (2.1)
No treatment	95 (40.3)
Number of hepatic metastases	2 (1-11)
Bilobar distribution	76 (32.5)
Size of metastases (cm)	3 (0.3-12)
Associated disease	164 (72.2)
Anesthetic risk	
ASA Ⅰ-Ⅱ	150 (63.6)
ASA Ⅲ-Ⅳ	86 (36.4)
Previous treatment of metastases	62 (27.3)
Preoperative CEA (ng/dL)	10.35 (0.4-3203)
Extrahepatic invasion	11 (4.8)

Intraoperative results

217 patients (92%) underwent IOUS. We found a higher amount or more invasive hepatic lesions than in the preoperative evaluation in 30% of the patients, leading to non-resection in half of these patients. 38 patients were found to have extrahepatic disease at the time of laparotomy, and in 18 of them a curative resection with resection of extrahepatic disease could be achieved. 159 patients (72%) underwent another procedure associated with liver resection, mainly cholecystectomy (105 patients) but also including colectomy (11 patients), splenectomy (one patient) and diaphragmatic and vascular resection (five patients) (Table 2).

Postoperative results (Table 3)

Pathological data: Non-involved margins (defined as the absence of tumor at any edge of the resection piece at the pathological examination) were achieved in 75.8% of patients. Extrahepatic invasion on pathological

Table 2 Operative characteristics of the patients *n* (%)

Characteristics	
Intraoperative ultrasonography	92%
Number of metastases (median, range)	2 (0-15)
Extrahepatic invasion	38 (16.1)
Type of resection	
Major hepatectomy	51 (21.6)
Minor hepatectomy	66 (28)
Atypical hepatectomy	36 (15.3)
Combined hepatectomy	36 (15.3)
No resection	47 (19.9)
Resection of extrahepatic disease	18 (7.5)
Peritoneal disease	4
Diaphragmatic invasion	4
Local disease (colon/rectum)	4
Hilar lymph node invasion	3
Inferior vena cava invasion	2
Splenectomy	1
Additional procedure	159 (72)
Blood loss (mL) (median, range)	370 (0-2500)
Vascular inflow exclusion	56.8%
Vascular exclusion time (min)	33 (4-128)
Need of transfusion	82 (36.8)
Surgery time (min)	220 (30-420)

analysis was found in 12.3% of patients. When comparing the number of nodules found at pathological examination with the ones preoperatively diagnosed, 28% of patients showed more nodules, whereas only 18.2% of patients had more nodules at pathological examination compared with the number diagnosed intraoperatively by IOUS.

Clinical data: Global postoperative mortality in the series was 1.7%. Minor postoperative complications were described in 41.1% of the patients in the series, with nine patients suffering biliary leak, nine patients having postoperative hepatic failure and only one case of postoperative bleeding. 4.7% of patients had major postoperative complications but only eight patients needed reoperation (4 due to intestinal fistula or perforation, 2 due to wound evisceration and one due to postoperative bleeding and infection).

Outpatient data: After resection, chemotherapeutic adjuvant treatment (based mainly on 5-fluorouracil alone or combined with irinotecan or oxaliplatin) was given to 52.9% of the patients.

Recurrence analysis

Tumor recurrence rate at 1, 3 and 5 years was 40.9%, 66% and 70.4% with a median tumor-free survival of 1.42 (0.4-1.9) years (Figure 1). When performing unifactorial analysis, preoperative extrahepatic disease (P = 0.02), CEA levels over 20 ng/dL (P = 0.017), nodules larger than 5 cm at pathological examination (P = 0.043) and extrahepatic disease at pathological examination (P = 0.009) were associated with a higher recurrence. Multivariate recurrence analysis showed that patients with preoperative extrahepatic disease (HR 3.355, P = 0.023) and CEA levels over 20 ng/dL (HR 1.812, P = 0.013)

Table 3 Postoperative results of patients undergoing curative resection *n* (%)

Postoperative results	
Number of metastases (median, range)	2 (1-18)
Size of metastases (cm) (median, range)	3.25 (0.7-15)
Pathological extrahepatic invasion	22 (12.3)
Margins	
> 1 cm	33.5%
< 1 cm	42.3%
Invaded	24.2%
Hospital stay (d)	9 (4-43)
Postoperative mortality	4 (1.7)
Major postoperative morbidity	11 (4.7)
Postoperative treatment	100 (52.9)
Neoplastic recurrence at 1 yr	77 (40.9)

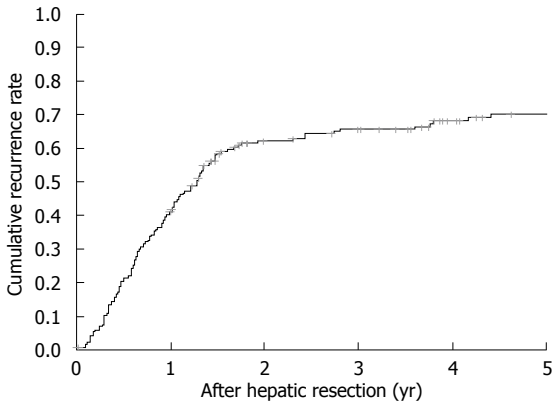

Figure 1 Neoplastic recurrence after hepatic resection.

before resection were exposed to a higher recurrence risk. No differences were observed when comparing patients by preoperative number and size of nodules and their lobar distribution. An affected surgical margin was not associated with higher recurrence compared to non-affected margins. When analyzing intraoperative (blood loss, need of blood transfusion, use of Pringle maneuver, type of resection) and postoperative events (biliary leak, postoperative complications, postoperative liver failure) only biliary leakage was associated with an increase in 5-year recurrence rate (0.9% *vs* 9.5%, P = 0.009) but without differences in survival rates.

Global survival and unifactorial survival analysis

The global survival at 1, 3 and 5 years in patients undergoing hepatic resection was 91%, 54% and 47%, respectively (Figure 2). Median survival was 3.6 years. Patients undergoing curative hepatic resection had better survival compared to patients in whom a curative resection was not possible (Figure 3). No factors associated with primary CRC tumor were found to make significant differences to patients' survival, with no differences in survival between patients with synchronous and metachronous CLM. When diagnosed with CLM, the presence of preoperative extrahepatic disease conferred worse survival compared to patients without extrahepatic disease (P = 0.0002) without influence by number and size of CLM. Patients with

Table 4 Time-related changes by period analysis *n* (%)

	First period (Jan 1994-Jan 2000)	Second period (Feb 2000-Jan 2006)	*P*
Patients (male:female)	93 (63:30)	143 (90:53)	NS
Age (yr) (mean, range)	63.9 (40-81)	62.5 (36-81)	NS
1-yr survival rate	88.3%	85.6%	NS
1-yr recurrence rate	38.5%	44%	NS
Anesthetic risk			
ASA Ⅰ-Ⅱ	60 (64.5)	90 (62.9)	NS
ASA Ⅲ-Ⅳ	33 (35.5)	53 (37.1)	NS
Number nodules	1.77 (1-6)	2.3 (1-11)	0.012
Size of nodules	3.8 (0.8-11)	3 (0.2-12)	NS
Bilobar disease	19 (20.4)	57 (39.9)	0.002
Extrahepatic disease	1 (1.1)	10 (7)	0.03
Preoperative CEA level (ng/dL)	13.5 (0.4-3203)	8.9 (0.6-1715)	0.03
Adjuvant treatment to CLM	13 (14)	49 (34.3)	0.001
Resectability rate	69.9%	86.7%	0.002
Use of intraoperative US	91.3%	92.9%	NS
Concordance of IOUS	56.5%	67.9%	NS
Procedures performed			0.024
Major hepatectomy	23 (35.4)	28 (22.6)	
Minor hepatectomy	18 (27.7)	48 (38.7)	
Atypical hepatectomy	17 (26.2)	20 (16.1)	
Combined hepatectomy	7 (10.8)	28 (22.6)	
Operative time (min)	226.7 ± 58.8	251 ± 65.3	0.01
Blood loss (mL)	440 (25-1700)	560 (80-2500)	NS
Need of transfusion	40%	44.7%	NS
Complications rate	32.3%	60.5%	0.001
Minor complications	24.6%	54%	0.001
Major complications	7.7%	5.6%	NS
Postoperative stay (d)	11 (5-34)	9 (4-43)	0.018
Postoperative mortality	1.5%	1.6%	NS
Complementary treatment of CLM	60%	49.2%	NS

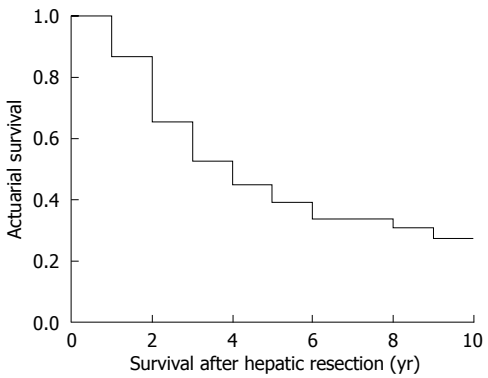

Figure 2 Global survival post resection.

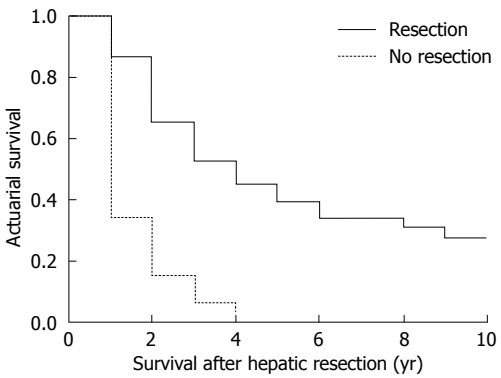

Figure 3 Survival for type of surgery.

a preoperative CEA value under 20 ng/dL had better survival compared to patients with CEA over 20 ng/dL (P = 0.035). When analyzing pathological results of CLM, the presence of more than four nodules (P = 0.015) and extrahepatic invasion (P = 0.0044) was associated with worse survival. As with recurrence, intraoperative and postoperative events had no significant effect on survival.

Multivariate survival analysis

Regarding factors associated with primary CRC tumor, nodal invasion was the only factor accounting for a decreased survival (HR 1.743, P = 0.049). As in the unifactorial survival analysis, preoperative extrahepatic invasion was a significant factor in multivariate analysis (HR 3.223, P = 0.036). After resection, having only one nodule on pathological analysis was a protective factor for survival (HR 2.122, P = 0.042), whereas having nodules over 5 cm was a risk factor for a worse prognosis (HR 2.222, P = 0.049).

Time-related results analysis (Table 4)

No differences in overall 1- and 3-year survival (88.3% and 51.9% *vs* 85.6% and 52.3%) and 1-year recurrence (38.5% *vs* 44%) were observed between patients in the first time period when compared to patients in the second time period.

When analyzing preoperative factors, patients in the second group had a higher number of CLM, but no differences were found when comparing CLM size in

the two groups. Also, patients with bilobar disease and presence of extrahepatic disease were more frequent in the second period. CEA levels were higher in the first period. Adjuvant treatment prior to CLM resection was more frequently used in the second period.

Resectability rate was higher in the second period. The frequency of IOUS use did not differ between periods, and there was only a non-significant trend of better accuracy in the second period. Changes in the distribution of surgical techniques were observed with a higher amount of minor hepatectomies and combined procedures in the second period. Operative time in resected patients was slightly higher in the second period, while there were no differences in blood loss and in the perioperative transfusion rate.

No differences were observed in postoperative mortality between the two periods. Postoperative complications were more often observed in the second period with increased minor complications and a similar major complications rate. Hospital stay was shorter in the second group. A similar amount of patients received complementary treatment after CLM resection.

DISCUSSION

At the present time, surgery remains the only curative treatment for CLM[1,5,6]. Advances in liver surgery, perioperative care, radiological techniques and the introduction of new chemotherapeutical agents have greatly changed resection strategies and have increased the number of patients in whom curative resection can be achieved[1,12-15].

Our series show that the majority of patients had good prognostic preoperative characteristics according to CRS (few and small nodules, unilobular distribution and CEA under 20 ng/dL). Such a patient selection can be a reason to explain a high CLM resection rate achieved (80%), comparable to other major series[19], which enhances our policy of feasibility of resection of CLM with extension of traditional criteria. This preoperative selection would not be complete without the use of IOUS as previously recommended by many authors[20-22]. In our series IOUS showed a clear selection benefit as it detected a preoperative underdiagnosis of CLM leading to non-resection in nearly 15% of the patients. For this reason, we strongly advocate IOUS exploration as a compulsory adjunct prior to liver resection for CLM.

The presence of extrahepatic disease is one of the classical contraindications to CLM resection, a belief which has changed as surgical expertise has made it possible to perform curative resections including all extrahepatic disease[1]. Despite this positive aspect, it has to be noted that the preoperative and the postoperative (pathological) presence of extrahepatic disease in patients with CLM is associated with a higher neoplastic recurrence and a worse patient survival[9,23]. This fact should raise concern regarding need for a stricter patient selection when extrahepatic disease is found on preoperative imaging, as only curative resection is a valid option for these patients[24]. Also, even though no conclusive data exist at the present time, the finding of intraoperative extrahepatic disease probably deserves a closer postoperative follow-up with a more aggressive use of complementary chemotherapeutical treatment.

Definition of an adequate minimal surgical margin when resecting CLM remains an unresolved issue[25,26]. At the present time the ideal margin is yet to be defined as some authors have shown that negative margins of either 1-4 mm, 5-9 mm or up to 1 cm have similar overall recurrence rates and survival[25-27]. In our study differences in recurrence and survival could not be found when comparing free surgical margins under and above 1 cm, a fact that supports these previous observations. Interestingly, a positive surgical margin was not associated with worse survival or higher recurrence in our series, which could be explained by the concept that the really important margin would be the one which remains in the patient, as some studies have pointed[28].

The presence of CLM has been historically linked to a low overall survival, although in recent years the advances in imaging, chemotherapeutical agents and surgical techniques have increased the survival rates, approaching a 5-year survival of 60%[12-15]. In our series 5-year survival in resected patients was 47%, which can be positively compared with other major hepatobiliary center series, although some of these series do not reflect the surgical and perioperative improvements achieved in the last decade[29].

Up to 50% of patients with resected CLM will develop recurrence of neoplastic disease, the majority of them in the first 2 years, and this fact remains the most determinant factor for patient survival[30-34]. Several recurrence-associated factors such as size and number of CLM, stage of the primary tumor, CEA levels, disease-free interval and resection margin have been described; these being the basis for the clinical scores which are used for predicting recurrence and thus survival in patients with CLM[9-11]. In our study we were only able to find preoperative CEA above 20 ng/dL and extrahepatic invasion as significant factors that would be associated with an increased recurrence rate.

Despite the already known effect of preoperative factors, some authors have also pointed to the influence of intraoperative and postoperative events on recurrence and survival[35,36]. Improvements in surgical technique and perioperative management have lead to a decrease in postoperative mortality in major centers, under 5% in the last few years, clearly improving prognosis in patients with CLM[37]. In our series global postoperative mortality was 1.7%, a similar rate when compared to other series from high-volume centers[29]. Interestingly, when analyzing postoperative complications, only biliary leakage was associated with a significantly increased first-year recurrence rate, but had no influence on differences in survival rate. This data should be taken with caution as more studies need to be done in order to confirm this unexpected and difficult to explain observation, but it raises concern about the influence of postoperative events in the prognosis of CLM patients.

Surgical treatment of CLM has been challenged with

advances in surgical techniques and better perioperative management in recent times[29,32,36,37]. Our series failed to show an improvement with time in short- and medium-term survival and recurrence as these two parameters did not improve in the second time period. However, similar survival and recurrence rates between the two periods should not be seen as a negative fact because patient conditions could also have changed (and not necessarily improved) with time. In fact, even though no differences in patient basal status were observed in the second period, an increase of unfavorable prognostic factors (higher number of CLM, bilobar distribution, presence of extrahepatic disease) could be found. This extension of indications for CLM resection would be mainly responsible for the limitation of the expected effects of improved surgical experience and use of better technology when resecting CLM. Also, adjuvant treatment of CLM was more frequent in the second period, a fact related to the presence of synchronous (another indicator of bad prognosis) or initially unresectable CLM, situations that would limit improvement in overall survival rates in the second period, as seen in our study.

Resectability rate is said to depend basically on good patient selection and surgical expertise[19], a fact that seems to be confirmed by our series as resectability rate increased with time. Also, the sensitivity for diagnosing CLM with IOUS has increased with time probably as a result of the availability of higher definition instruments and the experience gained by surgeons with this technique[38]. However, preoperative imaging techniques have also improved their limits for CLM diagnosis with time. This would help explain the fact that in our series the concordance rate of preoperative studies and IOUS showed a positive trend with time, although it did not reach significance due to better accuracy in both IOUS and preoperative staging tools[39]. However, and as stated before, sufficient reasons do not exist at the present time to limit IOUS in the staging of CLM.

Our series shows an increase in global postoperative complications with time without any differences in mortality. While mortality rates could be expected to stay the same or decrease due to improved surgical expertise and perioperative care, despite more difficult resections[32,34,36], this increase in complications can be explained easily when dividing them into major and minor events[40]. Major complications are closely related to mortality and for this reason it would be expected that they did not change over time. However, minor complications mainly influence hospital stay and this latter parameter decreased with time in our series. The rationale behind this is that improved awareness and means for detection of minor complications are implemented with time, which would invariably result in better treatment of these complications.

To conclude, it can be stated that despite the extension of indications for resective surgery in the second time period, with an inclusion of patients having more unfavorable prognostic factors, improvements in surgical technique, adjuvant treatments and preoperative imaging have played an important role in avoiding a greater mortality compared to the past. As surgery remains the only curative treatment, a careful patient selection and a judicious use of adjuvant therapies prior to and after surgery are crucial to continue offering patients with CLM a real chance of a cure.

COMMENTS

Background

Although many prognostic factors for survival and neoplastic recurrence of patients undergoing surgical treatment for colorectal liver metastases (CLM) are already identified, the effects of time-related changes in these patients are still poorly studied because of the presence of many involved factors.

Research frontiers

Prognostic factors implicated in survival and recurrence for patients undergoing surgical treatment for CLM are important in order to select better treatment options in these patients.

Innovations and breakthroughs

The observed effects of time-related changes in morbidity, mortality, overall survival and neoplastic recurrence in patients with CLM result from the inclusion of patients with unfavorable prognostic factors and the recent improvements in preoperative imaging, surgical technique and adjuvant treatments.

Applications

A careful patient selection and judicious use of adjuvant therapies prior to and after surgery are crucial for continuing to improve prognosis in patients with CLM.

Peer review

The manuscript retrospectively reviewed patients who underwent hepatic resection for liver metastases from colorectal cancer, identified prognostic factors for recurrence and survival after hepatic resection, and speculated as to time-related advances of this important disease. The paper is a well-designed work which aims to evaluate the influence of some factors on the recurrence and survival of patients who undergo CLM resection and, to compare these items between two periods.

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Promoter methylation and mRNA expression of *DKK-3* and *WIF-1* in hepatocellular carcinoma

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Abstract

AIM: To investigate the promoter methylation status and mRNA expression of *DKK-3* and *WIF-1* gene in hepatocellular carcinoma (HCC).

METHODS: *DKK-3* and *WIF-1* acted as Wnt-antagonists and tumor suppressors, but hypermethylation of the gene promoter and low mRNA expression activated Wnt signaling aberrantly and induced the development of HCC. Methylation status of the *DKK-3* and *WIF-1* gene promoter was investigated using methylation specific polymerase chain reaction (PCR) in tumor and adjacent non-cancerous tissues from 33 HCC patients and 20 normal liver tissues served as control. The expression of *DKK-3* and *WIF-1* mRNA was also determined by real-time quantitative reverse transcriptase PCR. The relationship between methylation, mRNA expression, and clinical data, as well as methylation and mRNA expression of the two genes were analyzed.

RESULTS: The methylation of *DKK-3* and *WIF-1* genes in HCC increased significantly compared with adjacent non-cancerous tissues and normal control tissues (χ^2 =7.79, $P < 0.05$; χ^2 = 4.89, $P < 0.05$), and no significant difference in methylation between adjacent non-cancerous tissues and normal control tissues was observed. In HCC tissues, significant differences in the *DKK-3* promoter methylation were observed in age and cirrhosis, and significant differences of the *WIF-1* promoter methylation were observed in HBsAg and cirrhosis. The average expression of *DKK-3* mRNA in HCC and adjacent non-cancerous tissues was increased significantly compared with normal control tissues. The average expression of *WIF-1* mRNA showed no significant difference among the three tissues. The mRNA expression of *DKK-3* gene in HCC was decreased as the pathological grade increased.

CONCLUSION: The aberrant promoter methylation and decreased expression of *DKK-3* and *WIF-1* may be an important mechanism in HCC, and may be a far-reaching significance in early diagnosis and therapy of HCC.

Key words: Hepatocellular carcinoma; *DKK-3*; *WIF-1*; Promoter methylation

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most fatal human malignancies and the third most frequent cause of tumor-related death[1]. However, the molecular mechanisms of hepatocarcinogenesis remain unclear. At present, α-fetoprotein (AFP) is used as a tumor marker in the screening of HCC, but it is only sensitive to advanced liver cancer or as a marker of the recurrence after hepatectomy. Therefore, identifying a tumor marker for HCC is important for early diagnosis and therapy.

The Wnt/β-catenin protein signal transduction pathway participates in early embryonic development. *DKK-3* and *WIF-1* are two key genes in the Wnt signal

transduction pathway, and the subnormal function of *DKK-3* and *WIF-1* can contribute to the activation of the Wnt pathway and result in carcinogenesis through dysregulation of cell proliferation and differentiation. CpG islands are short fragments of DNA that contain a higher CG frequency than other regions. The hypermethylation of CpG islands is associated with transcriptional repression of these genes. Aberrant promoter hypermethylation of genes occurs frequently during the pathogenesis of human cancers, and has been found to be a primary mechanism in the down-regulation of these genes. Methylation-specific PCR (MSP) and real-time quantitative reverse transcriptase polymerase chain reaction (RT-PCR) can detect these epigenetic changes and can be used for cancer detection.

In the present study, MSP and real time quantitative RT-PCR were used to investigate the methylation status and the expression of *DKK-3*, and *WIF-1* in patients with HCC, to explore the potential carcinogenesis of HCC and to investigate its early diagnostic and therapeutic potential.

MATERIALS AND METHODS

Tissue samples

Thirty-three samples of HCC and adjacent non-cancerous tissues (> 2 cm away from tumor) were obtained from the Department of Surgery, First Affiliated Hospital of Anhui Medical University between July 2006 and July 2007. These samples were documented through a pathology laboratory database. The age of the 33 patients (27 male, 6 female) ranged between 18 and 67 years, with a median age of 51 years. Ten patients were Edmonson stage Ⅰ, 18 patients were stage Ⅱ, and five patients were stage Ⅲ. The diameter of the tumor was smaller than 3 cm in five patients, and ⩾ 5 cm in 28 patients. Portal vein tumor thrombus was found in three patients. As a control, 20 samples of normal liver tissues were collected from the resection of hemangiomas between July 2006 and July 2007. The samples were snap-frozen in liquid nitrogen and stored at -80℃ until the extraction of DNA and RNA.

Nucleic acid extraction

Genomic DNA and total RNA were extracted from HCC tissues, adjacent non-cancerous and normal control tissues using the Qiagen kit (Qiagen). The concentration of DNA and RNA were determined with a spectrophotometer and their integrity was assessed by gel electrophoresis.

MSP

We investigated whether the promoter regions of the two genes were methylated. Genomic DNA was modified with sodium bisulfite using the CpGenome DNA Modification kit (Intergen) according to the specifications of the manufacturer. MSP was performed in a total volume of 25 μL, containing 2 μL modified template DNA, AmpliTaq Gold (Roche 5 U/μL) 0.2 μL, 10 × buffer 2.5 μL, dNTP (10 μmol/L) 1 μL, each primer (10 μmol/L) 0.5 μL, Mg^{2+} (25 μmol/L) 2 μL, and RNase free water 16.3 μL. MSP reactions were subjected to an initial incubation at 95℃ for 10 min, followed by 35 cycles of 95℃ for 45 s, and annealing at the 52-57℃ for 45 s and 72℃ for 45 s. Final extension was completed by incubation at 72℃ for 5 min. Primer sequences of MSP and unmethylation-specific PCR (USP) are shown in Table 1[2]. MSP products were separated on 2% agarose gels and visualized after ethidium bromide staining. The product bands of MSP and USP were calculated using a TANON GIS gel image analysis system, and the relative methylation level was determined by MSP ratio = MSP band density/(MSP band density + USP band density).

Real- time quantitative RT-PCR

Total RNA (1 μg) was reverse-transcribed with RevertAid First Strand cDNA Synthesis Kit (Fermentas) in a final volume of 20 μL. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The cDNAs of interest were quantified by real-time quantitative PCR using the Rotor-Gene 3000. Samples were assayed in a 25 μL reaction mixture containing 1 μL of cDNA, 12.5 μL of 2 × QuantiTect probe PCR Master Mix (Qiagen), 1 μL of each specific primer, 0.5 μL of fluorogenic probe and 9 μL of RNase free water. The sequence of the primer and probe is shown in Table 1. Real-time quantitative RT-PCR reactions were subjected to initial incubation at 95℃ for 10 min, followed by 35 cycles of 95℃ for 15 s, and 60℃ for 60 s.

Statistical analysis

Statistical analyses were performed using SPSS software version 13.0. χ^2 test, t test, non-parameter Spearman test and Fisher's exact test were used to compare the relationship of methylation and clinical data. One-year disease-free survival was analyzed through the log rank test. The level for a statistically significant difference was set at $P < 0.05$ for all the tests.

RESULTS

MSP

Methylation status of *DKK-3* and *WIF-1* in the three tissues: The methylation status of *DKK-3* and *WIF-1* in the three kinds of tissues is shown in Table 2 and Figure 1. In HCC and adjacent non-cancerous tissues, the methylation rate of *DKK-3* was 37.61% ± 4.26% and 15.96% ± 3.91%, respectively. The methylation status of *WIF-1* was 29.70% ± 3.24% in HCC and 18.34% ± 4.02% in adjacent non-cancerous tissues. The methylation rate of *DKK-3* and *WIF-1* in HCC was higher than that in adjacent non-cancerous tissues ($P < 0.05$). The methylation of *DKK-3* and *WIF-1* was not observed in normal control tissues. There was no linear correlation between methylation of *DKK-3* and *WIF-1* ($r = 0.296$, $P > 0.05$).

Table 1 Methylation-specific PCR, real-time PCR primer, probe sequence and annealing temperature

Gene	Primer purpose	Primer sequence (5'--3')	Annealing temperature (℃)	Product size (bp)
DKK-3	MSP-M	F: GGGGCGGGCGGCGGGGC	58	120
		R: ACATCTCCGCTCTACGCCCG		
	MSP-U	F: TTAGGGGTGGGTGGTGGGGT	56	126
		R: CTACATCTCCACTCTACACCCA		
	Real time RT-PCR	F: GTAAGTTTCCCCTCTGGCTTG	60	90
		R: AAGCACCAGACTGTGAAGCCT		
		Probe: FAM+AGGTGTTGTGCATTTGTTCAGCTCCC+TAMRA		
WIF-1	MSP-M	F: CGTTTTATTGGGCGTATCGT	57	145
		R: ACTAACGCGAACGAAATACGA		
	MSP-U	F: GGGTGTTTTATTGGGTGTATTGT	52	154
		R: AAAAAAACTAACACAAACAAAATACAAAC		
	Real time RT-PCR	F: TCCAAACACCTCAAAATGCTATC	60	119
		R: GAACCCATCAGGACACTCGC		
		Probe: FAM+ACAAGCTGAGTGCCCAGGCGG+TAMRA		
GAPDH	Real time RT-PCR	F: CCACTCCTCCACCTTTGAC	60	102
		R: ACCCTGTTGCTGTAGCCA		
		Probe: FAM+TTGCCCTCAACGACCACTTTGTC+TAMRA		

M: Methylation; U: Unmethylation; F: Sense primer; R: Antisense primer.

Table 2 Methylation level of *DKK-3* and *WIF-1* in two tissues (mean ± SE, %)

Methylation level	*DKK-3*	*WIF-1*
HCC tissues	37.61 ± 4.26	29.70 ± 3.24
Adjacent non-cancerous tissues	15.96 ± 3.91	18.34 ± 4.02
	$P < 0.05$	$P < 0.05$

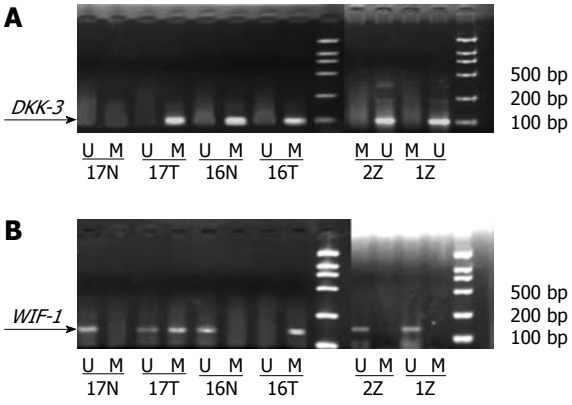

Figure 1 *DKK-3* (A) and *WIF-1* (B) MSP electrophoretogram. T: HCC tissues; N: Adjacent non-cancerous tissues; Z: Control tissues; M: Methylation-specific PCR products bands; U: Unmethylation-specific PCR products bands.

Relationship of methylation and clinical data: The relationship between *DKK-3* and *WIF-1* methylation and clinical data such as sex, age, Child-Pugh score, AFP, HBsAg, tumor number, cirrhosis, pseudo-capsule and pathology class is shown in Tables 3 and 4. The methylation rate of the *DKK-3* gene was higher in older (≥ 60) than younger (< 60) patients, higher in non-cirrhosis than that in cirrhosis patients ($P < 0.05$), and higher in HBsAg (-) than in HBsAg (+) patients. There was no relationship and no associations observed between the methylation status of *DKK-3* and *WIF-1* in adjacent non-cancerous tissues and clinical data ($P < 0.05$).

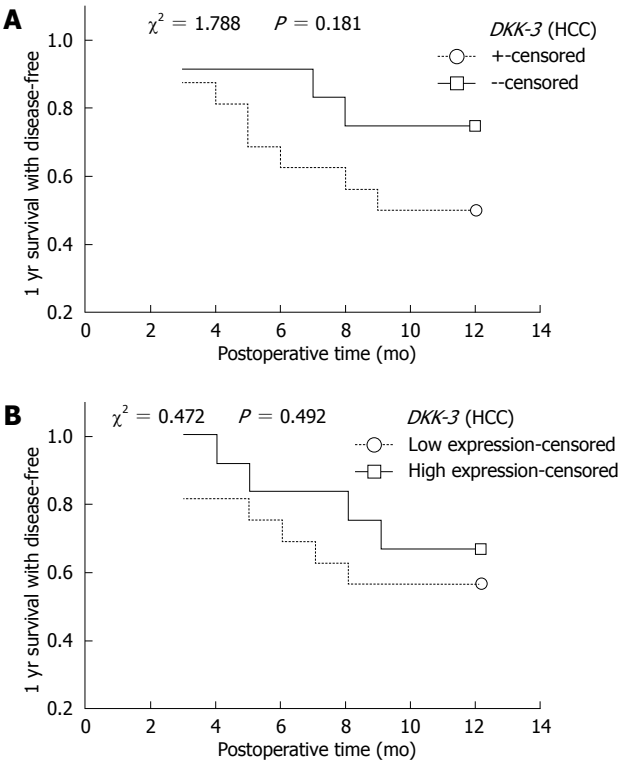

Figure 2 Relationship between methylation (A) and mRNA expression (B) of *DKK-3* gene and 1-year disease-free survival in HCC tissues.

Relationship of methylation and 1-year disease-free survival: Complete follow-up data was obtained from 27 (27/33) cases of HCC, and the follow-up time was > 1 year. Among the 27 cases, 11 cases showed disease recurrence or died. Kaplan-Meier method and Log-rank test were used to calculate the survival rate and the significance of difference. One-year disease-free survival decreased as time passed, but there was no significant difference in the *DKK-3* or *WIF-1* between HCC and adjacent non-cancerous tissues (Figure 2).

Table 3 Methylation and clinical data of *DKK-3* genes in HCC and adjacent non-cancerous tissues

Clinical data	Methylation in HCC			Methylation in adjacent non-cancerous tissues		
	Negative	Positive	*P*	Negative	Positive	*P*
Sex						
Male	12	15	1.000	21	6	1.000
Female	3	3		5	1	
Age (yr)						
< 60	13	7	0.011	17	3	0.393
⩾ 60	2	11		9	4	
Child						
A	12	17	0.308	23	6	1.000
B	3	1		3	1	
AFP (ng/mL)						
< 20	7	10	0.308	15	3	0.674
⩾ 20	8	8		11	4	
Number of tumor						
Single	11	13	1.000	20	4	0.358
Multiple	4	5		6	3	
Tumor diameter (cm)						
< 3	2	3	1.000	3	2	1.000
⩾ 3	13	15		23	5	
HBsAg						
(+)	15	14	0.108	23	6	1.000
(-)	0	4		3	1	
Hepatocirrhosis						
(+)	15	10	0.004	20	5	1.000
(-)	0	8		6	2	
False capsule						
(+)	14	14	0.346	23	5	0.282
(-)	1	4		3	2	
Tumor thrombus						
(+)	1	2	1.000	3	0	1.000
(-)	14	16		23	7	
Edmonson stage						
Ⅰ / Ⅱ	13	15	1.000	22	6	1.000
Ⅲ	2	3		4	1	

Table 4 Methylation and clinical data of *WIF-1* genes in HCC and adjacent non-cancerous tissues

Clinical data	Methylation in HCC			Methylation in adjacent non-cancerous tissues		
	Negative	Positive	*P*	Negative	Positive	*P*
Sex						
Male	17	10	0.659	23	4	1.000
Female	3	3		5	1	
Age (yr)						
< 60	7	5	1.000	17	3	1.000
⩾ 60	13	8		11	2	
Child						
A	17	12	1.000	24	5	1.000
B	3	1		4	0	
AFP (ng/mL)						
< 20	11	8	1.000	16	3	1.000
⩾ 20	9	5		12	2	
Number of tumor						
Single	14	10	1.000	21	3	0.597
Multiple	6	3		7	2	
Tumor diameter (cm)						
< 3	2	3	1.000	5	0	0.569
⩾ 3	18	10		23	5	
HBsAg						
(+)	20	9	0.017	24	5	1.000
(-)	0	4		4	0	
Hepatocirrhosis						
(+)	18	7	0.035	21	4	1.000
(-)	2	6		7	1	
False capsule						
(+)	18	10	0.360	24	4	1.000
(-)	2	3		4	1	
Tumor thrombus						
(+)	1	2	0.547	2	1	0.400
(-)	19	11		26	4	
Edmonson stage						
Ⅰ / Ⅱ	16	12	0.625	23	5	0.569
Ⅲ	4	1		5	0	

Real-time quantitative RT-PCR

mRNA expression of *DKK-3* and *WIF-1*: The *DKK-3* mRNA expression in HCC, adjacent non-cancerous tissues and normal control tissues was 0.773 ± 0.319, 0.833 ± 0.316 and 1.012 ± 0.125, respectively. The expression of *DKK-3* mRNA in HCC and adjacent non-cancerous tissues was significantly lower than that in normal control tissues ($P < 0.05$). There was no significant difference between HCC and adjacent non-cancerous tissues. *WIF-1* mRNA expression in the three tissues was 0.853 ± 0.510, 0.820 ± 0.316 and 0.995 ± 0.148, without significant difference among them. Pearson's product-moment correlation analysis showed that there was no correlation between *DKK-3* and *WIF-1* in HCC tissues ($r = 0.472$, $P = 0.127$).

Relationship between *DKK-3* and *WIF-1* mRNA expression and clinical data: The relationship between *DKK-3* and *WIF-1* mRNA expression and clinical data are shown in Tables 5 and 6. *DKK-3* mRNA expression in HCC was decreased as the pathological grade increased ($P < 0.05$), but no significant correlation was found with other clinical data. The expression of *DKK-3* mRNA in adjacent non-cancerous tissues, and the expression of *WIF-1* mRNA in HCC and adjacent non-cancerous tissues showed no significant correlation with clinical data ($P > 0.05$). Pearson's product-moment correlation analysis showed that there was no correlation between *DKK-3* and *WIF-1* in HCC, adjacent non-cancerous tissues and normal control tissues ($P > 0.05$).

Relationship of mRNA expression and 1-year survival: The expression of *DKK-3* and *WIF-1* gene mRNA in HCC and adjacent non-cancerous tissues was divided into two groups: higher expression group (> average expression) and lower expression group (⩽ average expression). Kaplan-Meier curve showed that the disease-free survival was decreased. The survival rate of the lower expression group was lower than that of the higher expression group, but without significant difference ($P > 0.05$, Figure 2).

Relationship between methylation and mRNA expression: The relationship between mRNA expression and methylation status in HCC and adjacent non-cancerous tissues is shown in Figure 3, and the relationship of *DKK-3* and *WIF-1* mRNA expression from HCC and adjacent non-cancerous tissues was not found between methylation and non-methylation ($P > 0.05$).

Table 5 mRNA expression and clinical data of *DKK-3* in HCC and adjacent non-cancerous tissues (mean ± SE)

Clinical data	Case	The mRNA expression of *DKK-3* in HCC	*t*	*P*	The mRNA expression of *DKK-3* in adjacent non-cancerous tissues	*t*	*P*
Sex							
Male	27	0.776 ± 0.346	0.153	0.880	0.779 ± 0.319	0.888	0.404
Female	6	0.761 ± 0.168			0.913 ± 0.338		
Age (yr)							
< 60	20	0.756 ± 0.356	0.334	0.741	0.819 ± 0.341	0.195	0.847
⩾ 60	13	0.795 ± 0.285			0.840 ± 0.274		
Child							
A	29	0.761 ± 0.331	0.789	0.466	0.829 ± 0.329	0.171	0.869
B	4	0.863 ± 0.225			0.813 ± 0.155		
AFP (ng/mL)							
< 20	17	0.716 ± 0.287	1.069	0.294	0.840 ± 0.354	0.124	0.902
⩾ 20	16	0.835 ± 0.384			0.826 ± 0.281		
Number of tumor							
Single	24	0.740 ± 0.315	0.971	0.348	0.823 ± 0.298	0.262	0.797
Multiple	9	0.860 ± 0.329			0.860 ± 0.377		
Tumor diameter (cm)							
< 3	4	0.908 ± 0.300	0.943	0.399	0.792 ± 0.352	0.290	0.780
⩾ 3	29	0.755 ± 0.322			0.841 ± 0.315		
HBsAg							
(+)	29	0.660 ± 0.233	0.984	0.372	0.817 ± 0.304	0.639	0.563
(-)	4	0.789 ± 0.329			0.955 ± 0.418		
Hepatocirrhosis							
(+)	25	0.780 ± 0.319	0.046	0.964	0.761 ± 0.268	2.157	0.057
(-)	8	0.774 ± 0.365			1.060 ± 0.361		
False capsule							
(+)	28	0.757 ± 0.326	1.750	0.132	0.819 ± 0.307	0.790	0.466
(-)	5	1.014 ± 0.299			0.966 ± 0.395		
Tumor thrombus							
(+)	3	0.770 ± 0.330	0.738	0.519	0.811 ± 0.307	1.088	0.379
(-)	30	0.890 ± 0.260			1.057 ± 0.379		
Edmonson stage							
Ⅰ / Ⅱ	28	0.807 ± 0.335	3.380	0.020	0.820 ± 0.317	0.564	0.596
Ⅲ	5	0.586 ± 0.350			0.910 ± 0.333		

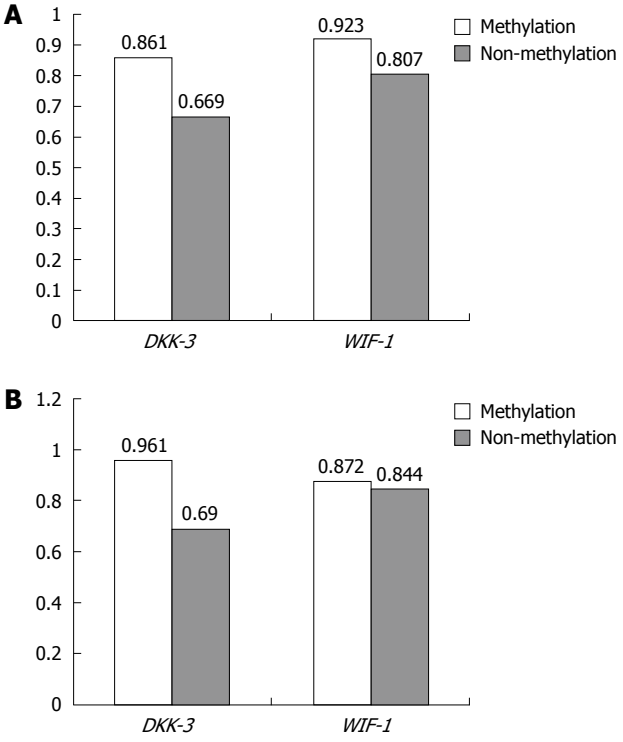

Figure 3 Relationship between methylation and mRNA expression of *DKK-3* and *WIF-1* in HCC (A) and adjacent non-cancerous tissues (B). $P > 0.05$.

DISCUSSION

The Wnt signal transduction pathway plays a very important role in embryonic development, and abnormalities may lead to developmental defects and cellular malignant transformation[3]. It has been shown that disturbances of the Wnt signal transduction pathway were significantly related to human neoplastic transformation. Overexpression of *Wnt* genes has been reported in many cancers, including breast cancer, esophagus cancer, colorectal cancer, malignant melanoma, leukemia, prostate cancer, endometrial carcinoma, HCC, thyroid cancer, and pancreatic cancer[4,5]. Gene silencing related with the hypermethylation of its promoter is an important epigenetic mechanism, and an important mechanism to regulate the expression of genes in the body.

The *DKK-3* gene locus maps to 11p15.1 of the human chromosome, which encodes a Wnt complex receptor antagonist. Wnt will combine with Frizzle receptor and activate Wnt signaling if the DKK-3 protein is inactivated. Promoter-hypermethylation and reduced expression of the *DKK-3* gene were found in bladder cancer, lung cancer cell lines and tissues[2,6]. In our experiment, the methylation status of *DKK-3* in HCC was significantly higher than that in adjacent non-

Table 6 mRNA expression and clinical data of *WIF-1* in HCC and adjacent non-cancerous tissues (mean ± SE)

Clinical data	Case	The mRNA expression of *WIF-1* in HCC	*t*	*P*	The mRNA expression of *WIF-1* in adjacent non-cancerous tissues	*t*	*P*
Sex							
Male	27	0.882 ± 0.542	0.942	0.365	0.828 ± 0.320	0.286	0.783
Female	6	0.720 ± 0.336			0.787 ± 0.318		
Age (yr)							
< 60	12	0.767 ± 0.397	1.092	0.289	0.793 ± 0.247	0.551	0.589
⩾ 60	21	0.985 ± 0.643			0.862 ± 0.407		
Child							
A	29	0.854 ± 0.543	0.067	0.948	0.793 ± 0.309	1.243	0.286
B	4	0.845 ± 0.171			1.015 ± 0.337		
AFP (ng/mL)							
< 20	19	0.873 ± 0.516	0.231	0.819	0.828 ± 0.377	0.148	0.883
⩾ 20	14	0.831 ± 0.519			0.812 ± 0.247		
Number of tumor							
Single	24	0.927 ± 0.532	1.292	0.212	0.801 ± 0.288	0.496	0.630
Multiple	9	0.704 ± 0.408			0.872 ± 0.393		
Tumor diameter (cm)							
< 3	5	1.002 ± 0.750	0.507	0.636	0.796 ± 0.415	0.147	0.889
⩾ 3	28	0.826 ± 0.469			0.825 ± 0.304		
HBsAg							
(+)	29	0.875 ± 0.527	0.155	0.886	0.838 ± 0.312	0.754	0.497
(-)	4	0.820 ± 0.681			0.695 ± 0.360		
Hepatocirrhosis							
(+)	25	0.840 ± 0.523	0.260	0.797	0.810 ± 0.322	0.331	0.746
(-)	8	0.893 ± 0.486			0.853 ± 0.314		
False capsule							
(+)	28	0.860 ± 0.490	0.152	0.886	0.816 ± 0.296	0.144	0.892
(-)	5	0.812 ± 0.676			0.846 ± 0.454		
Tumor thrombus							
(+)	3	0.815 ± 0.505	1.392	0.276	0.806 ± 0.319	0.840	0.474
(-)	30	1.223 ± 0.481			0.960 ± 0.300		
Edmonson stage							
Ⅰ / Ⅱ	28	0.910 ± 0.517	1.005	0.083	0.821 ± 0.308	0.141	0.894
Ⅲ	5	0.532 ± 0.360			0.848 ± 0.401		

cancerous tissues and normal control tissues, and the expression of *DKK-3* mRNA in HCC and adjacent non-cancerous tissues was significantly lower than that in normal control tissues. These results show that the methylation of the *DKK-3* gene promoter plays an important role in the carcinogenesis of HCC. It has been reported that hypermethylation was frequently present in elderly people[7,8], and HCC was reported to be closely linked to hepatitis B (HBV) infection. The results of previous reports are consistent. Our results show that hypermethylation was more frequent in elderly people (age ⩾ 60 years) and non-cirrhotic HCC tissues, which suggests that the silence of the *DKK-3* gene resulted from the hypermethylation of its promoter. The expression of *DKK-3* mRNA in Edmonson stage Ⅲ was significantly lower than that in stage Ⅰ / Ⅱ, suggesting that the expression of *DKK-3* is negatively related to the stage of tumor and cell proliferation. Therefore, the decreased expression of *DKK-3* mRNA may affect the invasion and intrahepatic metastasis of HCC. In addition, the expression of *DKK-3* was the lowest in late G1 of the cell cycle[9].

The *WIF-1* gene locus maps to 12q14.1 of the human chromosome, and encodes a conservative secreted protein that inhibits the activity of Wnt by holding back the regular or non-regular Wnt signal transduction pathway. Promoter-hypermethylation and reduced expression of the *WIF-1* gene was found in lung cancer cell lines and tissues, malignant pleural mesothelioma cell lines and tissues, and nasopharyngeal cancer cell lines[10-12]. Our study showed that the methylation status of *WIF-1* in HCC was higher than that in adjacent non-cancerous tissues and normal control tissues, and was higher in the HBsAg (-) and non-cirrhosis patients, which implies that the *WIF-1* gene silence related to promoter hypermethylation may represent the pathogenesis with HBV infection that results in HCC development. *WIF-1* mRNA expression in HCC and non-cancerous tissues was not significantly lower than that of normal control tissues, which may be attributed to gene silence of multiple factors. *WIF-1* mRNA expression has no relationship with clinical data, suggesting that HCC development is a complex polygene and multipathway process[13].

In summary, Wnt signaling is a major cell signal transduction pathway, and plays an important role in the proliferation and differentiation of cells. Wnt-antagonists function as tumor suppressors and contribute to the pathogenesis of several human malignancies. In the normal state, DKK-3 and WIF-1 can act as negative regulators of Wnt signaling, however, hypermethylation of the gene promoter and

low expression of mRNA will activate Wnt signaling aberrantly, and induce the development of HCC. At the same time, hypermethylation of the *DKK-3* gene in the elderly and non-cirrhotic HCC, and hypermethylation of the *WIF-1* gene in HbsAg (-) and non-cirrhotic HCC suggests that there may be two independent mechanisms in the carcinogenesis of HCC. One is HBV infection, which induces HCC development, and the other is the aberrant expression of Wnt-antagonist genes, such as *DKK-3* and *WIF-1*, which induces HCC development. A synergistic action of these factors has not been identified, and further study is needed. Our results may provide a reliable way to improve the early diagnosis of HCC and new therapies by blocking this pathway in the treatment of HCC.

COMMENTS

Background

The Wnt signal transduction pathway is significantly related to human neoplastic transformation. The Wnt-antagonist genes function as tumor suppressors and contribute to the pathogenesis of several human malignancies. Recently, promoter CpG hypermethylation and gene silencing in *DKK-3* and *WIF-1* genes have been identified in several human malignancies. The *DKK-3* gene locus maps to 11p15.1 of the human chromosome, which encodes a Wnt complex receptor antagonist. Wnt will combine with Frizzle receptor and activate Wnt signaling if the DKK-3 protein is inactivated. The *WIF-1* gene locus maps to 12q14.1 of the human chromosome, which encodes a conservatively secreted protein that inhibits the activity of Wnt by holding back regular or non-regular Wnt signal transduction pathway.

Research frontiers

Recently, Wnt antagonists have received increasing attention because of their potential role in carcinogenesis. Many studies have reported a relationship between hypermethylation of a gene promoter and low expression of mRNA in several human carcinomas. Promoter hypermethylation and reduced expression of the *DKK-3* gene were found in bladder cancer, lung cancer cell lines and tissues.

Innovations and breakthroughs

Few studies have described the correlation between Wnt antagonists and the development of hepatocellular carcinoma (HCC). The results of this study suggest that *DKK-3* and *WIF-1* may act as negative regulators of Wnt signaling and may be related to the development of HCC. However, this pathogenesis is different from that of hepatitis B virus infection inducing HCC development.

Applications

In this study, the authors investigated the methylation status and mRNA expression of *DKK-3* and *WIF-1* in HCC, adjacent non-cancerous tissues and normal control tissues, and found that the aberrant Wnt antagonists play an important role in carcinogenesis of HCC. This finding may provide a reliable way to improve early diagnosis of HCC and new therapies by blocking this pathway in the treatment of HCC.

Terminology

MSP: Methylation-specific polymerase chain reaction is a simple, rapid and inexpensive method to determine the methylation status of CpG islands. This approach allows the determination of methylation patterns from very small samples of DNA and can be used in the study of abnormally methylated CpG islands in neoplasia.

Peer review

Promoter-hypermethylation and the reduced expression of *DKK-3* and *WIF-1* were shown to be important mechanisms of hepatocarcinogenesis. These results may provide a reliable way to improve early diagnosis of HCC and develop new therapies by blocking this pathway in the treatment of HCC.

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ORIGINAL ARTICLES

Silencing of signal transducer and activator of transcription 3 expression by RNA interference suppresses growth of human hepatocellular carcinoma in tumor-bearing nude mice

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Abstract

AIM: To explore the effect of silencing of signal transducer and activator of transcription 3 (STAT3) expression by RNA interference (RNAi) on growth of human hepatocellular carcinoma (HCC) in tumor-bearing nude mice *in vivo*.

METHODS: To construct the recombinant plasmid of pSilencer 3.0-H1-STAT3-siRNA-GFP (pSH1-siRNA-STAT3) and establish the tumor-bearing nude mouse model of the HCC cell line SMMC7721, we used intratumoral injection together with electroblotting to transfect the recombinant plasmid pSH1-siRNA-STAT3 into the transplanted tumor. The weight of the nude mice and tumor volumes were recorded. *STAT3* gene transcription was detected by semi-quantitative reverse transcription polymerase chain reaction (RT-PCR). Level of protein expression and location of STAT3 were determined by Western blotting and immunohistochemical staining. STAT3-related genes such as survivin, c-myc, VEGF, p53 and caspase3 mRNA and protein expression were detected in tumor tissues at the same time. The terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was used to detect apoptosis of tumor cells.

RESULTS: The weight of the treated nude mice increased, and the tumor volume decreased markedly compared with those of the mock-treated and negative control groups ($P < 0.01$). The results of RT-PCR and Western blotting showed that mRNA and protein levels of STAT3 declined markedly in the treated group. The change in STAT3-related gene expression in tumor tissues at the mRNA and protein level also varied, the expression of survivin, VEGF and c-myc were obviously reduced, and expression of p53 and caspase3 increased ($P < 0.01$). Most of the tumor tissue cells in the treated group developed apoptosis that was detected by TUNEL assay.

CONCLUSION: Silencing of STAT3 expression by RNAi significantly inhibits expression of STAT3 mRNA and protein, and suppresses growth of human HCC in tumor-bearing nude mice. The mechanism may be related to down-regulation of survivin, VEGF and c-myc and up-regulation of p53 and caspase3 expression. Accordingly, the *STAT3* gene may act as an important and effective target in gene therapy of HCC.

Key words: RNA interference; Signal transducer and activator of transcription 3 transcription factor; Hepatocellular carcinoma; Xenograft model antitumor assays; Nude mouse

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INTRODUCTION

RNA interference (RNAi) is a post-transcriptional gene-silencing mechanism, in which the homologous RNA sequences are introduced into the cells that inhibit the

expression of a particular gene, through the introduction of short interfering RNAs[1]. There have been a large number of confirmed reports that RNAi targeting oncogenes may successfully inhibit the growth of tumor cells *in vitro* and *in vivo*. Hence, RNAi has been turned into a potent technology for tumor therapy.

Hepatocellular carcinoma (HCC) research has shown that RNAi targeting genes are related to initiation, development and metastasis of HCC[2-4]. Signal transducer and activator of transcription 3 (STAT3) is an important member of the family of STAT. Its signal pathway is closely associated with the proliferation, differentiation and apoptosis of cells, and constant activation of STAT3 can promote cell proliferation and carcinogenesis. At present, STAT3 is defined as an oncogene[5]. Studies of STAT3 have focused on a variety of tumor cell lines, including leukemia, multiple myeloma, breast cancer, prostate cancer, melanoma tumor, and lung cancer, while HCC has been rarely investigated. Persistently activated STAT3 is detected in many HCC cell lines and tissues.

Our preliminary research revealed that the recombination plasmid pSH1Si-STAT3 targeting the *STAT3* gene could dramatically inhibit the expression of STAT3 mRNA and protein in the HCC cell line SMMC7721 and the proliferation of HCC cells, and induce apoptosis of HCC cells[6]. Therefore, we designed the present experiment to verify the therapeutic effect of recombination plasmid pSH1-siRNA-STAT3 in HCC *in vivo*.

MATERIALS AND METHODS

Human HCC cell lines

The human HCC cell line SMMC7721 was kept in the Department of Pathophysiology of the School of Basic Medicine in Jilin University, and cultured in Iscove's Modified Dulbecco's Medium (Gibco, BRL, USA) supplemented with 10% fetal bovine serum (Gibco, BRL), in an incubator containing 5% CO_2 at 37℃, and then digested and passaged with 0.25% trypsin.

Animals

Male BALB/c nude mice were purchased from the Institute of Zoology, Chinese Academy of Sciences. They were 5-wk-old, SPF grade and weighed 17.76 ± 1.83 g, and were fed in an aseptic laminar flow room at 25℃ and 60%-70% humidity, with a standard rodent diet and water.

Construction of recombination plasmid

The plasmids pSilencer3.0-H1-STAT3-siRNA and pSilencer1.0-U6-STAT3-siRNA-GFP were kindly provided by the Research Center of Prostate Diseases in Jilin University. The sequences of STAT3-siRNA were: 5'-GCAGCAGCTGAACAACATGTTCAAGAGACA TGTTGTTCAGCTGCTGCTTTTT-3' (forward), and 5'-AATTAAAAAGCAGCAGCTGAACAACATGTCT CTTGAACATGTTGTTCAGCTGCTGCTGCGGCC-3' (reverse). The two plasmids were linearized with *Eco*RI and *Hin*dII, and then pSilencer3.0-H1 and STAT3-siRNA-GFP were combined to form pSilencer3.0-H1-STAT3-siRNA-GFP (pSH1Si-STAT3), which was cloned and sequenced. A negative control pSH1Si-Scramble, which has no significant homology with human gene sequences, was constructed by the same method. The extraction of plasmids followed the routine Fastfiler Endo-free Plasmid Maxiprep protocol (BioDev, Beijing, China).

Cell preparation

The cells in logarithmic growth phase were digested with 0.25% trypsin and centrifuged. The cells were collected and washed three times with PBS, and the living cells were counted under a microscope, and adjusted to a final concentration of 2.5×10^7 cells, with PBS.

Establishment of tumor-bearing nude mouse model

The nude mice were fed on the super-clean biological laminar flow shelf for 1 wk. When the weight of the mice increased to 21.02 ± 1.81 g, tumor cells were injected subcutaneously into their hindquarters. Each mouse was seeded with 200 μL that contained 5×10^6 cells. Then, the mice were observed daily for mental state, diet and stools, and were monitored every 4 d for tumor size and body weight. The model was established successfully, when there were nodules with the size of a grain of rice after 1 wk, and a volume of 50-70 mm^3 after 2 wk. We measured the largest (a) and smallest (b) superficial diameter with Vernier calipers and calculated the tumor volume (V) = $a \times b^2 \times 0.5236$.

Treatment of tumor-bearing nude mice

The nude mice were divided into mock-treated, negative control and treated groups of five mice each. PBS was used for transfection in the mock-treated group, pSH1Si-Scramble was used for transfection in the negative control group, and pSH1Si-STAT3 was used for transfection in the treated group. The treatment volume was 40 μL and the plasmid concentration was 0.5 μg/μL, which was injected intratumorally at several points. At 30 s after injection, an electrical impulse was applied twice at intervals of > 1 min. Electrodes were placed at both ends of the tumor long and short axes; the electric field strength was 200 V/cm, electric pulse duration was 50 ms, and pulse frequency was 1 Hz. This process was repeated on days 10 and 17. Therefore, tumor volume and mouse body weight were measured every 4 d. On day 20, the tumors were removed and their weight and volume were measured, and then each tumor was divided into two parts, one was used for immunohistochemical analysis, and the other was used for screening mRNA expression of STAT3 and related genes and protein expression.

Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR)

Tumor RNA was extracted using Trizol reagent (Gibco Life Technologies, Inc., Langley, OK, USA) following its manufacturer's instructions. The concentration of the total RNA was detected by UV spectrophotometry. RT-PCR was performed by the two-step method. Synthesis

of cDNA was performed using the cDNA Synthesis Kit (Genomy, Beijing, China). The conditions were: 94℃ for 5 min, 94℃ for 30 s, 56℃ for 30 s, 72℃ for 45 s, and 72℃ for 7 min, for 30 cycles; the total volume was 25 μL. For quantitative analysis of c-myc, p53, survivin, VEGF and caspase3 mRNA, the expression of the housekeeping gene β-actin was used as an internal standard. The primer sequences were: STAT3: sense 5'-TTGCCAGTTGTGGTGATC-3', antisense 5'-AGAC CCAGAAGGAGCCGC-3'; β-actin: sense 5'-AAGTAC TCCGTGTGGATCGG-3', antisense 5'-ATGCTATCA CCTCCCCTGTG-3'. The primers were synthesized by the Shanghai Sangon Biological Engineering Technology & Services Co. Ltd. The PCR fragments were separated and visualized in 20 g/L agarose gels stained with ethidium bromide. Semi-quantitative analysis was performed with the Gis gel analysis software (Shanghai, China). All experiments were done in triplicate. The ratio of the photo-density of the RT-PCR product of the target gene and β-actin was used to identify the expression intensity of the target gene.

Western blotting analysis

The tumor tissues were ground and sonicated with supersonic lytic buffer that contained 50 mmol/L NaH_2PO_4, 100 mmol/L Tris-HCl, 250 mmol/L NaCl, 100 mg/L PMSF, 1 mg/L Aprotinin, pH 8.0, and then centrifuged at 12000 × *g* for 40 min. The Bio-Rad standard curve was used to determine the concentration of protein in each lysate. Loading buffer was added to each lysate, which was subsequently boiled for 5 min and then electrophoresed by SDS-PAGE. The proteins were mixed with 2 × loading buffer with the same volume before electrophoresis. After transferring onto nitrocellulose, proteins were incubated with antibodies (anti-p-STAT3 and β-actin, purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA) and then with peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology). Detection was performed with an enhanced chemiluminescence agent. The analysis was performed with the Bandscan analysis software (Sterling, VA, USA). All experiments were done in triplicate. The ratio of the proteins of STAT3 and β-actin was used to identify the expression intensity.

Immunohistochemical analysis

The tumor tissues were embedded in paraffin and then sliced. The slices were stained with hematoxylin and eosin (HE) and the expression of p-STAT3 protein (Santa Cruz Biotechnology) was determined using the streptavidin biotin complex immunohistochemical staining kit (Boster, Wuhan, China). A positive outcome was brown staining in the cytoplasm or nucleus, and the ratio of positive to total cells was > 15%.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay

The tumor tissues were fixed with 10% formalin for 4 h and then embedded in paraffin. The slices were deparaffinized in water and placed in 3% H_2O_2 for 10 min at room temperature. The TUNEL assay was carried out according to the manufacturer's instructions (KGI Biotechnology Company, Nanjing, China). A positive result was brown staining in the nucleus.

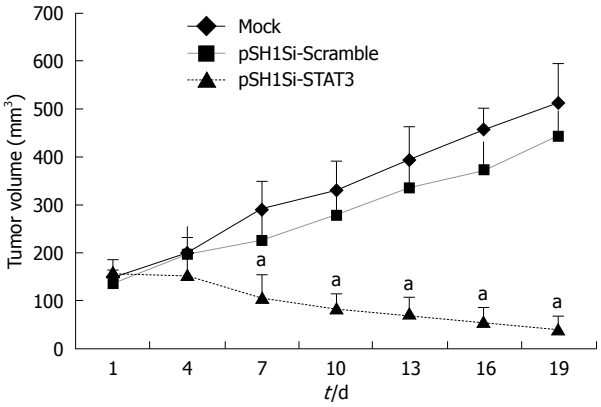


Figure 1 Growth curves of SMMC7721 tumor in nude mice in different treated groups. $^aP < 0.01$ *vs* pH1Si-Scramble group.

Statistical analysis

Statistical analysis was performed using SPSS 12.0 software (Chicago, IL, USA). The results were expressed as mean ± SD. The data were treated by Student's *t* test to determine statistical significance. $P < 0.05$ was considered statistically significant.

RESULTS

Therapeutic effect on tumor-bearing nude mice

The body weight of tumor-bearing nude mice decreased gradually and tumor volume increased in the mock-treated and pSH1Si-Scramble groups (Figure 1). However, the tumor volume in the pSH1Si-STAT3 group was markedly diminished from day 7, and body weight clearly increased from day 10 (Figure 2). Compared with the mock-treated and pSH1Si-Scramble groups, there were marked differences in the pSH1Si-STAT3 group ($P < 0.01$). Although the changes in the pSH1Si-Scramble group were less than those in the mock-treated group, there was no statistical significance ($P > 0.05$). During excision of subcutaneous tumor, we found that tumor volumes were larger and blood supplies were richer in the mock-treated and pSH1Si-scramble groups than those in the pSH1Si-STAT3 group (Figure 3), and the tumor weight of the three groups was 0.67 ± 0.07, 0.6 ± 0.07 and 0.18 ± 0.09, respectively. There were great differences between the pSH1Si-STAT3 and the mock-treated and pSH1Si-Scramble groups ($P < 0.01$), but differences between the mock-treated and pSH1Si-Scramble groups were not significant ($P > 0.05$).

Expression of STAT3 and related genes

The mRNA expression of the target genes was analyzed by RT-PCR. The mRNA levels of *STAT3*, *VEGF*, *survivin* and *c-myc* genes declined obviously, but mRNA

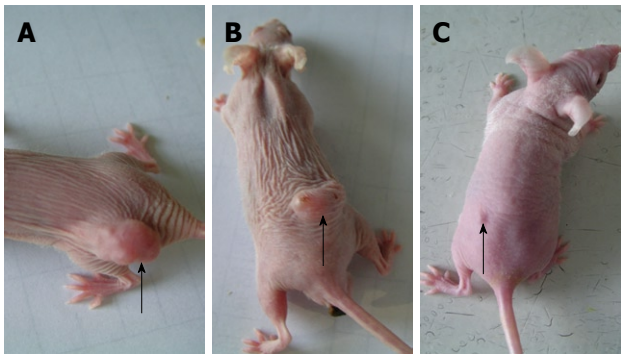


Figure 2 Comparison of tumor volume in nude mice in different treated groups. A: Mock-treated group; B: pSH1Si-Scramble group; C: pSH1Si-STAT3 group. Black arrow points to tumor tissue.

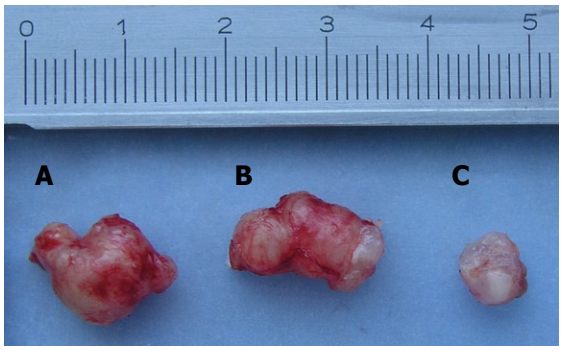
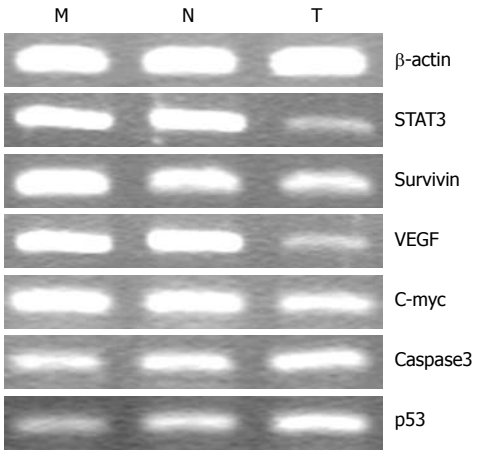


Figure 3 Tumor appearances in different treated groups. A: Mock-treated group; B: pSH1Si-Scramble group; C: pSH1Si-STAT3 group.



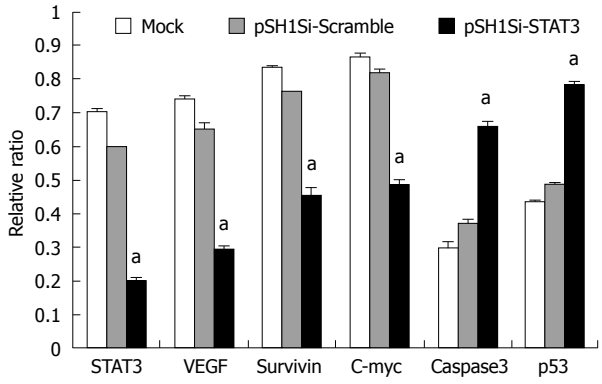
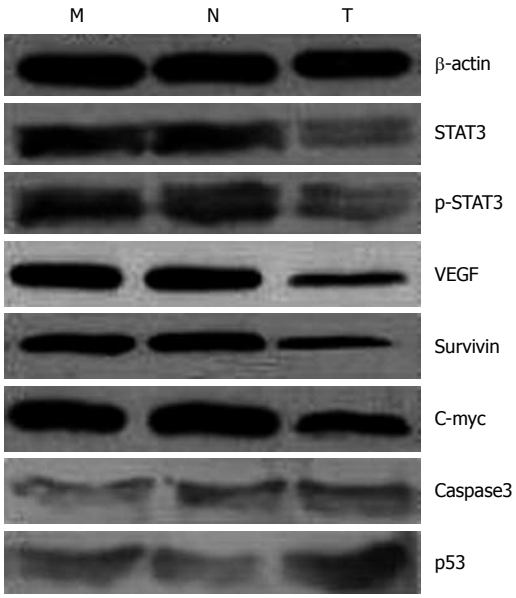


Figure 4 RT-PCR analysis of mRNA for *STAT3* and related genes in tumor tissues of different treated groups. M: Mock-treated group; N: pSH1Si-Scramble group; T: pSH1Si-STAT3 group. $^aP < 0.01$ *vs* pSH1Si-Scramble group.



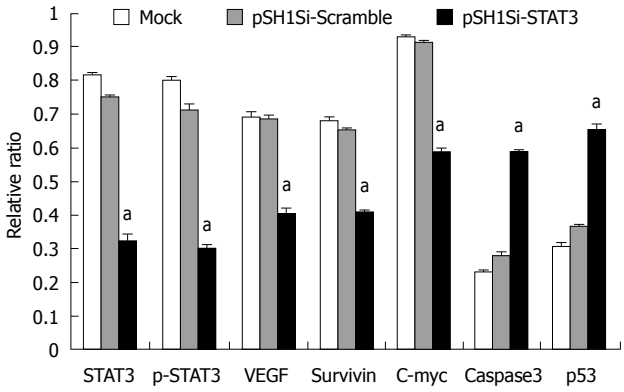


Figure 5 Western blotting analysis of STAT3 and related genes protein in tumor tissues in nude mice in different treated groups. $^aP < 0.01$ *vs* pSH1Si-Scramble group.

for the *caspase3* and *p53* genes in the pSH1Si-STAT3 group increased. The ratio of the photo-density of the RT-PCR product of the target genes and β-actin varied among the three groups, the differences between the pSH1Si-STAT3 and the mock-treated and pSH1Si-Scramble groups were significant ($P < 0.01$, Figure 4), but the difference between the mock-treated and pSH1Si-Scramble groups was not significant ($P > 0.05$).

Changes in protein expression of STAT3 and related genes

The protein expression of the target genes was analyzed by Western blotting. The protein levels of *STAT3*, *p-STAT3*, *VEGF*, *survivin* and *c-myc* genes were down-regulated clearly, but those of active *caspase3* and *p53* genes were up-regulated in the pSH1Si-STAT3 group. The ratio of the proteins of the target genes and β-actin varied among the three groups. The differences in the pSH1Si-STAT3 group were significantly greater than those in the mock-treated and pSH1Si-Scramble groups ($P < 0.01$, Figure 5), however, there was no significant difference between the mock-treated and pSH1Si-Scramble groups ($P > 0.05$).

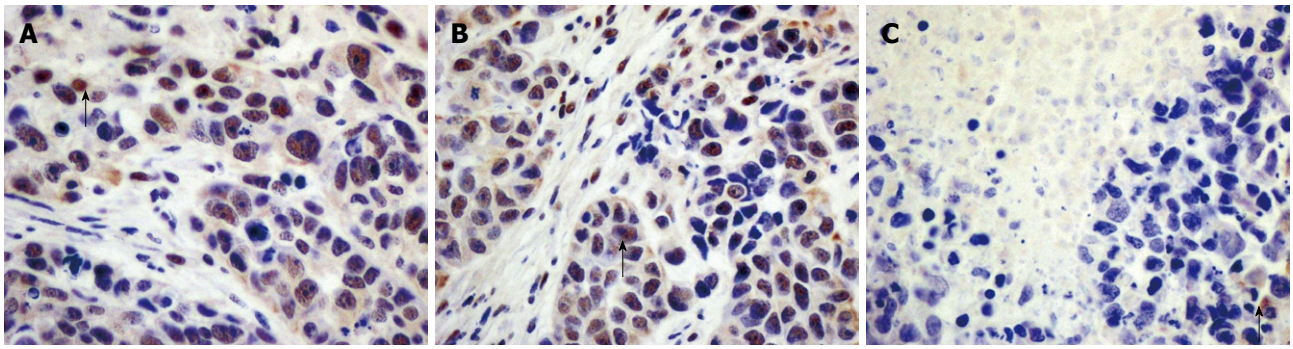


Figure 6 Detection of p-STAT3 protein expression by immunohistochemical assay of tumor tissues in nude mice in different treated groups (streptavidin biotin complex, × 400). A: Mock-treated group; B: pSH1Si-Scramble group; C: pSH1Si-STAT3 group. Black arrows point to positive cells, which were stained brown in the nucleus, using antibodies to p-STAT3.

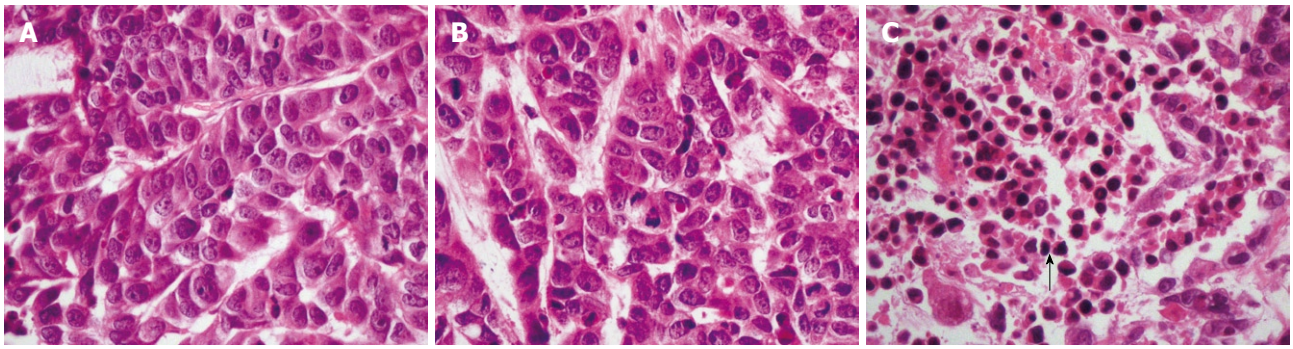


Figure 7 Tumor tissues in nude mice in different treated groups (HE, × 400). A: Mock-treated group; B: pSH1Si-Scramble group; C: pSH1Si-STAT3 group. Black arrow points to positive cells, which had karyopyknosis and red staining of the cytoplasm.

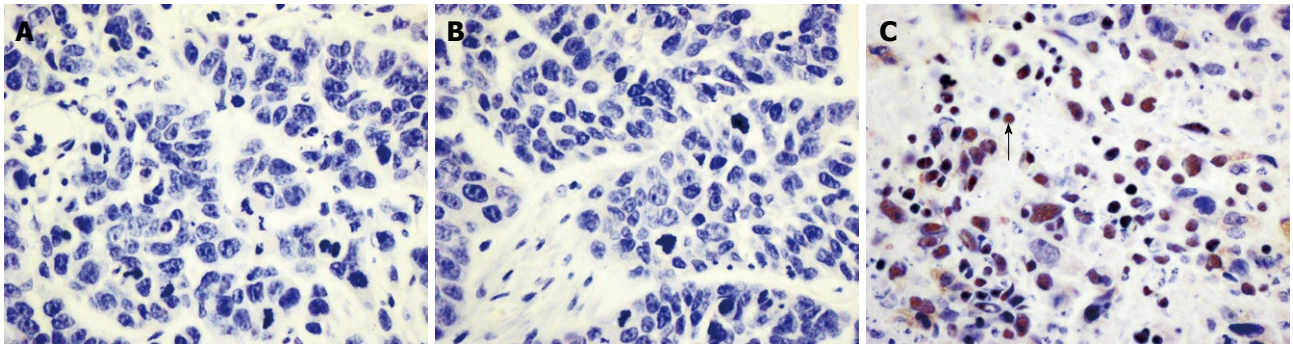


Figure 8 Detection of apoptosis with TUNEL assay in tumor tissues of tumor-bearing nude mice in different treated groups (× 400). A: Mock-treated group; B: pSH1Si-Scramble group; C: pSH1Si-STAT3 group, black arrow points to positive cells, in which the nuclei were stained brown.

Screening of STAT3 proteins

Immunohistochemistry showed that p-STAT3 was stained brown in the nuclei of many cells in the mock-treated and pSH1Si-Scramble groups, but were stained brown in the cytoplasm of only a few cells on the edge of tumors in the pSH1Si-STAT3 group (Figure 6).

Detection of apoptosis of tumor cells

The outcome of HE staining was observed under light microscopy (Figure 7). Many apoptotic cells were observed in the pSH1Si-STAT3 group, which showed karyopyknosis and red staining of the cytoplasm. There were also apoptotic cells in the pSH1Si-STAT3 group, in which the nuclei of many cells were stained brown (Figure 8).

DISCUSSION

There are many different genes and various signal transduction pathways involved in initiation and development of human HCC. The STAT3 and JAK/STAT3 signal transduction pathways are of considerable interest in tumors[7,8], and many studies[9,10] have detected the activation of STAT3 in many hepatocellular cell lines and HCC, and found that abnormal activation of STAT3 is related to the initiation and development of human liver cancer. It may be an effective strategy for HCC therapy to inhibit the abnormal expression of STAT3. At present, there are many methods[11-13] of targeting the *STAT3* gene, which include inhibiting indirectly

activation of STAT3 by receptor antagonist, neutralizing antibody, protein tyrosine kinase inhibitor and other agents, or blocking directly the functions of STAT3 by antisense oligonucleotides, decoy oligonucleotides, and RNAi. RNAi is a potent tool in tumor gene therapy, because of its high performance, specificity, convenient manipulation and low toxicity.

Our previous studies have demonstrated that the recombination plasmid pSH1Si-STAT3, which targets the *STAT3* gene, dramatically inhibits the expression of STAT3 mRNA and protein in the HCC cell line SMMC7721 *in vitro* and proliferation of HCC cells, and induces apoptosis of HCC cells[6]. Therefore, we designed this experiment in order to verify the therapeutic effect of recombination plasmid pSH1-siRNA-STAT3 in HCC *in vivo*. The present study indicated that body weight of mice was increased, and tumor volume was decreased. Tumor weight also declined in the pSH1Si-STAT3 group, and there were marked differences compared with that in the mock-treated and pSH1Si-Scramble groups. RT-PCR, Western blotting and immunohistochemistry showed that expression of mRNA and protein was markedly down-regulated, and there were many apoptotic cells, as shown by HE staining and TUNEL screening. These results verified that the recombination plasmid pSH1Si-STAT3 has a significant inhibitory effect on tumors in HCC-bearing nude mice.

At the same time, we detected the mRNA and protein levels of STAT3-related genes, and found that the expression levels of VEGF, survivin and c-myc were down-regulated clearly, but expression of caspase3 and p53 in pSH1Si-STAT3 was up-regulated. Compared with the mock-treated and pSH1Si-Scramble groups, the differences in expression were significant. Hence, we can infer that inhibiting the expression of STAT3 by siRNA in HCC may inhibit the activation of its target genes, recover the activity of some important anti-oncogenes, restrict the proliferation of tumor cells, and increase apoptosis. Therefore, it plays an important antineoplastic role.

The fate of c-myc attracted our attention. c-myc was not down-regulated by inhibition of the *STAT3* gene *in vitro*[6], but it was down-regulated *in vivo*. This may be related to the changed regulation mechanism of HCC cells after they have been removed from the body. It may also indicate that c-myc is not regulated directly by STAT3, but it is regulated by other genes that are targeted by STAT3. Hence, this mechanism of regulation requires further research.

Compared with Lipofectamine 2000 that we have used previously *in vitro*[6], which is characterized by high transfection efficiency and low toxicity, but high cost, the electrotransfection process used here *in vivo*, has the advantage of convenient operation, low cost, high efficiency and no toxicity. The results of the experiments demonstrated that the two methods of transfection obtained fine potency. Our study indicated that recombination plasmid pSH1-siRNA-STAT3 had significant anti-neoplastic activity *in vitro* and *in vivo*. A recombination plasmid that carries the same sequence of siRNA-STAT3 has a therapeutic effect on prostate tumor and melanoma[14,15], which leads us to conclude that one siRNA oligonucleotide drug may treat a variety of tumors. At the same time, one tumor can also be treated by several siRNA oligonucleotide drugs, and an optimal therapeutic effect may be obtained if multiple oncogenes are inhibited.

In our study, there were some changes after transfection in the pSH1Si-Scramble group, for example, changes in cell morphology and modification of gene expression. Although there was no significant difference compared with the mock-treated group, it suggested that the expression vectors that carried the scrambled sequence had some unknown effects on the cells, the mechanism of which needs further research.

We used intratumoral injection together with electrotransfection in our *in vivo* experiment, and obtained favorable results. Compared with intra-abdominal and intravenous injection, intratumoral injection is targeted and localized, allows direct injection of drugs into the feeding artery of the tumor, reduces the drug dose, and lengthens the dosage interval. However, this is an invasive procedure that requires skillful operators and special equipment, therefore, its application is limited. There are also potential dangers in the electrotransfection method, such as tissue injury or fibrosis where the electrode makes contact[16]. As a result, the key issues in the clinical application of RNAi are to develop vectors of high transfection efficiency and low toxicity, and to establish effective routes of administration.

In conclusion, the recombination plasmid pSH1-siRNA-STAT3 inhibited the growth of HCC cells *in vivo*, induced apoptosis, and had significant antineoplastic efficacy. RNAi that targets STAT3 has a clear therapeutic effect in HCC. Therefore, STAT3 may become an important target of biological therapy in HCC, which brings hope of clinical therapy using RNAi oligonucleotide drugs.

COMMENTS

Background

Hepatocarcinogenesis involves the mutation of multiple genes and alteration of the relevant signaling pathways. In recent years, signal transducer and activator of transcription 3 (STAT3) and related signaling pathways in liver carcinogenesis have attracted increasing attention. It has been shown that constitutively activated STAT3 is detected in many hepatocellular carcinoma (HCC) cell lines and tissues. This suggests that STAT3 is a promising molecular target for HCC gene therapy. Inhibition of abnormal expression of STAT3 may be an effective strategy for biological therapy of HCC.

Research frontiers

Some antineoplastic drugs, such as STAT3 inhibitors, tyrosine kinase inhibitor AG-490, micromolecular peptide and antisense oligodeoxynucleotides, have been used to target STAT3 *in vitro* and *in vivo* for HCC, however their efficacy is not satisfactory because of their poor specificity, rapid degradation, unsuitable molecular size, immunogenicity, poor ability to cross cell membranes, and need of special drug delivery mechanisms. RNA interference (RNAi) is a regulatory mechanism in most eukaryotic cells, which uses small double-stranded RNA (dsRNA) molecules to direct homology-dependent control of gene activity. As a result of the potency and specificity of RNAi, it has become an important tool to control gene expression through the introduction of siRNA.

Innovations and breakthroughs

Studies of targeting *in vitro* and *in vivo* over-expressed genes in HCC by RNAi, including c-myc, survivin, CCR1 and SMYD3, have been reported. However,

there has been still no report about targeting STAT3 by RNAi in HCC. In the present study, the authors used a DNA-vector-based RNAi approach to block STAT3 expression in HCC cells and tumor-bearing nude mice, to determine the role of constitutively activated STAT3 during HCC pathogenesis, and to explore the role and molecular mechanism of targeting STAT3 in HCC therapy.

Applications

By investigating the effect of silencing STAT3 expression by RNAi on the growth suppression and apoptosis of HCC cells, and the growth inhibition of HCC in tumor-bearing nude mice, this study may provides a new strategy for biological therapy of HCC by targeting STAT3. It may also offer a theoretical and experimental foundation for the clinical application of synthetic dsRNA-based RNAi.

Peer review

The manuscript by Li *et al* investigates the effects of silencing STAT3 on the growth of HCC *in vivo*. The authors present interesting and convincing data that silencing of STAT3 suppresses tumor growth *in vivo*. This is a good paper with very interesting data.

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Relationship between oxidative stress and hepatic glutathione levels in ethanol-mediated apoptosis of polarized hepatic cells

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Abstract

AIM: To investigate the role of reactive oxygen species (ROS) in ethanol-mediated cell death of polarized hepatic (WIF-B) cells.

METHODS: In this work, WIF-B cultures were treated with pyrazole (inducer of cytochrome P4502E1, CYP2E1) and/or L-buthionine sulfoximine (BSO), a known inhibitor of hepatic glutathione (GSH), followed by evaluation of ROS production, antioxidant levels, and measures of cell injury (apoptosis and necrosis).

RESULTS: The results revealed that ethanol treatment alone caused a significant two-fold increase in the activation of caspase-3 as well as a similar doubling in ROS. When the activity of the CYP2E1 was increased by pyrazole pretreatment, an additional two-fold elevation in ROS was detected. However, the CYP2E1-related ROS elevation was not accompanied with a correlative increase in apoptotic cell injury, but rather was found to be associated with an increase in necrotic cell death. Interestingly, when the thiol status of the cells was manipulated using BSO, the ethanol-induced activation of caspase-3 was abrogated. Additionally, ethanol-treated cells displayed enhanced susceptibility to Fas-mediated apoptosis that was blocked by GSH depletion as a result of diminished caspase-8 activity.

CONCLUSION: Apoptotic cell death induced as a consequence of ethanol metabolism is not completely dependent upon ROS status but is dependent on sustained GSH levels.

Key words: WIF-B cells; Alcohol; Fas/CD95; Glutathione; Caspase

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INTRODUCTION

It has been documented that the clinical progression of alcoholic liver disease (ALD) is associated with an increase in hepatocellular damage that may involve the promotion of apoptotic mechanisms[1]. As part of the effort to clarify mechanisms associated with ethanol-mediated hepatocellular apoptosis, researchers have utilized a variety of model systems (e.g. isolated hepatocytes or liver tissue from animals). Unfortunately, limitations exist with such models as the cells/tissue do not survive well or readily lose liver-specific functions in culture, reducing the effectiveness of identifying molecules and pathways that may be involved in hepatotoxic events. The aim of this study was to evaluate alcohol-mediated cellular alterations associated with

apoptotic mechanisms using a polarized hepatic (WIF-B) cell culture model.

The WIF-B cell is a cross between a human fibroblast (WI 38) and a Fao rat hepatoma cell[2]. This clone represents differentiated cells of hepatic origin that exhibit long-term viability in culture, develop a hepatocellular-polarized phenotype, and express genes coding for liver-specific proteins[3]. The use of WIF-B cells has recently emerged as an appropriate model for studying the effects of ethanol on cellular processes. Specifically, WIF-B cells endogenously express alcohol dehydrogenase (ADH) as well as CYP2E1 activity, allowing for the efficient metabolism of ethanol, and as a consequence, exhibit classic alcohol-mediated adverse effects such as triglyceride accumulation and apoptotic cell injury[4,5].

In general, the apoptotic cascade in hepatocytes can be triggered by signaling pathways that involve death receptor-mediated interactions and/or mitochondrial stress signals. These events result in the activation of cysteine proteases (caspases) which execute the proteolytic cleavage of proteins and the ultimate demise of the cell. In the case of ethanol-related cell death, several studies have demonstrated that the induction of caspases can be linked to the effects of various cytokines[6], the involvement of oxidative stress mechanisms[7], and glutathione depletion[8]. However, the contribution that ethanol and its metabolites make in enhancing reactive oxygen species (ROS) in WIF-B cells and what role oxidative stress plays in ethanol-mediated apoptosis in this model system is not known. Furthermore, discrepancies have been noted concerning the relationship that exists between antioxidant levels and hepatocellular damage associated with ALD, as GSH levels have been reported to be increased, decreased or unaltered following ethanol administration[9-11]. Additionally, it has been shown that the activation of apoptosis-executing caspases actually requires sustained glutathione levels as these proteases possess an essential cysteine thiol in the active site[12].

Therefore, this work examined the role that oxidative stress plays in ethanol-mediated apoptotic events in alcohol metabolizing cells which endogenously express ADH and CYP2E1, taking advantage of the ability to manipulate CYP2E1, ROS production, and antioxidant status in this culture model. Specifically, we examined hepatocellular death in ethanol-treated WIF-B cells by analyzing apoptosis along with correlative measurements of CYP2E1 expression, ROS production, and glutathione status.

MATERIALS AND METHODS

Materials

F-12 Coon's modified culture medium, 4-methylpyrazole (pyr), diallyldisulfide (DAS), N-acetylcysteine (NAC), antimycin A (AA), and actinomycin D (Act D) were obtained from Sigma Chemical Co. (St. Louis, MO). Fetal Bovine Serum (FBS) was obtained from Gemini Bio-Products (Woodland, CA). All other materials were of reagent grade.

Cell culture and treatments

WIF-B cells were cultured in F-12 Coon's modified media supplemented with 37.5 mL/L FBS as previously described[5]. Briefly, cells were cultured for 7-10 d to obtain maximal polarized phenotype followed by treatment with ethanol (25 and 50 mmol/L), 100 μmol/L DAS, or 5 mmol/L NAC for 48 h. CYP2E1 expression was induced by incubation with 0.25 mmol/L 4-methylpyrazole (pyr) for 4 d prior to ethanol and other treatments. GSH deficiency was induced in some cultures by adding 2 mmol/L buthionine-sulfoximine (BSO), an inhibitor of glutathione synthesis. Fas-mediated apoptosis was analyzed in cells treated with 25 mmol/L ethanol for 24 h prior to challenge with 0.5 g/L rabbit anti-rat Fas antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and 0.05 g/L actinomycin D.

The detection of oxidatively modified proteins

Protein oxidation was determined by detecting carbonyl groups within proteins using the Oxyblot® kit (Millipore, Temecula, CA) according to the manufacturer's instructions. Following derivatization with dinitrophenylhydrozone (DNP), modified proteins were detected by Western Blotting analysis using the Odyssey® Infrared Imaging System (LI-COR Biosciences, Lincoln, NE). Resolved proteins (10% SDS-PAGE), on nitrocellulose membranes were probed with 1:150 anti-rabbit DNP and 1:5000 mouse anti-GAPDH (Millipore, Temecula, CA), followed by goat anti-mouse and anti-rabbit Infrared IRDye®-labeled secondary antibodies (LI-COR).

Assessment of intracellular oxidative stress

The level of H_2O_2 was determined using the redox-sensitive dye 2',7'-dichlorodihydrofluorescein diacetate, H_2DCFDA (Invitrogen, Carlsbad, CA). Briefly, WIF-B cultures were incubated with 4 μmol/L H_2DCFDA for 30 min in the dark at the end of the treatment period and the oxidized fluorescent product detected (excitation 488 nm and emission 525 nm).

Analysis of CYP2E1 activity

Utilizing the p-nitrophenol oxidation assay as described[13], the activity of CYP2E1 was measured in microsomal proteins (0.05 g) isolated as previously noted[4].

Caspase activation assays

The activity of caspase-3 was evaluated by measuring the cleavage of the fluorogenic substrate Ac-DEVD-AMC (BD PharMingen, San Diego, CA) as previously described[5]. The activation of caspase-8 was determined using a Colorimetric Assay (R&D Systems Inc, Minneapolis, MN) following the manufacturer's instructions.

Flow cytometric determination of necrosis

The LIVE/DEAD® Fixable Cell Stain (Invitrogen, Carlsbad, CA) was used to determine necrosis based on the reaction of fluorescent reactive dye with intracellular

amines following entry through the compromised membranes of necrotic cells. Following staining with the reactive dye, the cells were analyzed by flow cytometry using the FACSCalibur (Becton Dickinson) with a 585/42 band pass filter. The results are expressed as the percentage of non-viable (necrotic) cells (reflected by the shift in mean fluorescent intensity) measured in the total cell population.

Glutathione determination

The detection of intracellular levels of reduced glutathione (GSH) in WIF-B cells was assessed using a commercial kit (Chemicon APT 250; Millipore, Temecula, CA) that detected the fluorescent product produced (excitation 380 nm and emission 461 nm) following the incubation of cell lysates with a monochlorobimane dye that has a high affinity for glutathione.

DNA fragmentation assay

DNA integrity was analyzed using the ApoTarget™ DNA Ladder kit, as described by the manufacturer (Invitrogen, Carlsbad, CA). Detached cells were collected by centrifugation (400 r/min) followed by DNA extraction. DNA fragments were resolved and visualized following agarose gel electrophoresis (5 EV/cm) and ethidium bromide staining. As a positive control, DNA was extracted from WIF-B cells that were induced to undergo apoptosis by UV treatment (320 μW/cm^2) as previously described[14].

Statistical analysis

Results refer to the average taken from three to seven experiments and are expressed as mean ± SE. Comparison of the values was performed using the Student *t* test with values, $P < 0.05$, considered significant.

RESULTS

Ethanol-mediated oxidative stress and its role in caspase activation

To evaluate the role of ethanol-mediated oxidative stress in the activation of apoptotic mechanisms in WIF-B cells, cultures were incubated with physiologically relevant concentrations (25 and 50 mmol/L) of ethanol in the presence or absence of DAS, and analyzed for the production of oxidative stress indices (protein carbonyl adduct formation and H_2O_2 generation) as well as caspase activation. It was determined that more cellular proteins were oxidatively modified as a result of ethanol treatment in comparison to control cells, and that the ethanol-mediated increase in carbonyl adducts was significantly reduced in the presence of DAS, an inhibitor of CYP2E1 (Figure 1A). In a similar fashion, using fluorescent spectrophotometry to detect the oxidized cleaved product, dichlorofluorescein (DCF), it was determined that ethanol treatment resulted in a two-fold increase in H_2O_2 production compared to untreated control cells (Figure 1B). Also, the presence of the antioxidant, NAC, provided

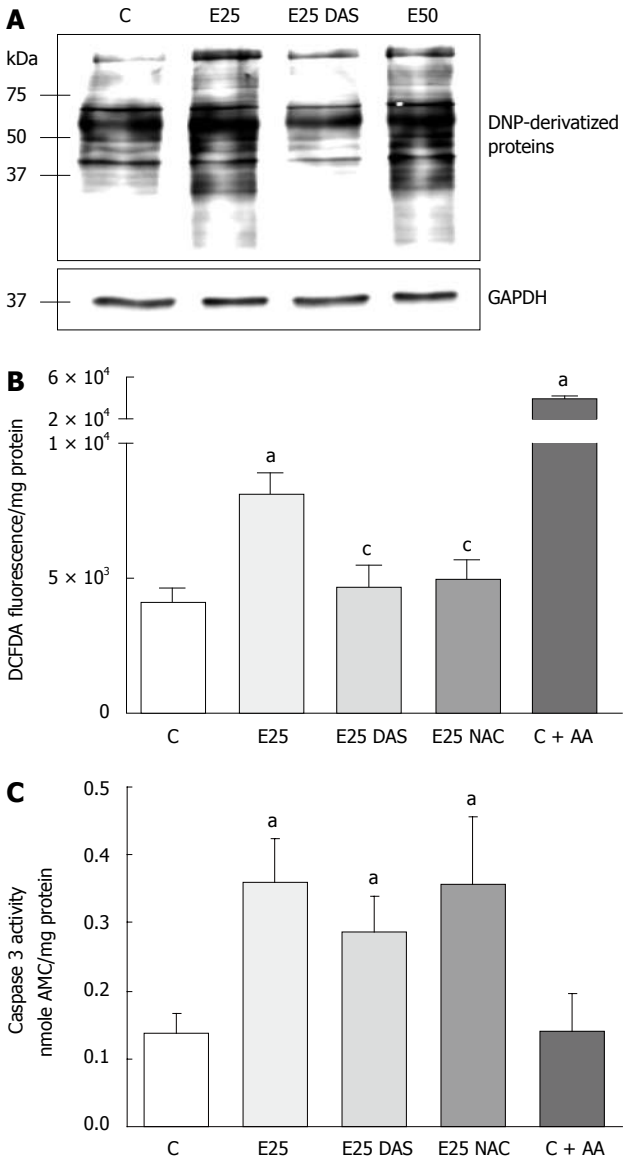

Figure 1 Ethanol-induced protein oxidation, oxidative stress, and caspase activation in WIF-B cells. WIF-B cultures were cultured for 48 h in the absence or presence of ethanol, and DAS or NAC when indicated. As a positive control for H_2O_2 production, some cultures were treated with 2 μmol/L Antimycin A (C + AA) for 30 min prior to analysis. A: Protein oxidation analysis represented as DNP-specific proteins detected in WIF-B cell lysates using the OxyBlot protein oxidation analysis; B: H_2O_2 production was detected by dichlorofluorescein fluorescence quantification. Data from 5 independent experiments is represented as the amount of fluorescent (DCFDA) oxidized products detected in the treated cells per mg protein; C: The activation of the proapoptotic protease, caspase-3, was detected as described in "Material and Methods" for five independent experiments. $^aP < 0.05$ *vs* control; $^cP < 0.05$ *vs* ethanol alone-treated cells.

significant protection against the ethanol-induced ROS elevation (Figure 1B). As a positive control for ROS production in these experiments, WIF-B cells were also incubated with AA, an inducer of H_2O_2 levels that resulted in a robust generation of DCF products as reflected by a 10-fold increase in ROS (Figure 1B). In contrast, when a measure of apoptosis (caspase activation) was conducted, it was determined that while ethanol treatment increased caspase-3 activity at a comparable two-fold elevation to that which was observed for

ethanol-related ROS production, the introduction of DAS or NAC did not provide any protection against ethanol-mediated apoptosis. Correspondingly, in AA-treated cells that represented an overproduction of ROS, no induction of caspase activity could be detected (Figure 1C).

Role of CYP2E1-dependent mechanisms in ethanol-mediated cell death of WIF-B cells

In light of the observations demonstrating that ethanol treatment results in the induction of ROS as well as apoptosis, while inhibitors of ROS could not attenuate caspase-3 activation, further evaluation of CYP2E1-dependent mechanisms was performed. First, it was determined that ethanol administration alone enhances CYP2E1 catalytic activity (Figure 2A) as well as protein expression (data not shown) in WIF-B cultures. Also, pretreatment of the cells with pyrazole for 4 d prior to ethanol challenge resulted in a further enhancement of CYP2E1 (Figure 2A). Utilizing this ability to manipulate the CYP2E1-ethanol metabolizing system, we next compared the production of ROS with caspase-3 activation in pyrazole-pretreated WIF-B cells with or without ethanol. The results indicate that when CYP2E1 was enhanced by pyrazole, a correlative 2-fold enhancement in ROS generation was observed when compared to ethanol-alone treated cells (Figure 2B). However, when the activity of caspase-3 was evaluated under the same conditions, it was determined that the pyrazole induction of CYP2E1 was not associated with an enhancement of apoptosis over what was observed in ethanol-alone treated cells (Figure 2B). Despite the fact that the activation of caspase-3 was not affected by enhanced ROS, the pyrazole and ethanol-treated cells displayed similar damaging morphological changes such as the loss of cell-cell contacts. Indeed, following evaluation of cell viability by flow cytometry, it was determined that when CYP2E1 expression and ROS were enhanced, the presence of non-viable cells significantly increased, demonstrating enhancement of necrotic cell death (Figure 2C). Correspondingly, AA treated cells (which display high ROS levels yet no caspase activation) presented morphologically as necrotic ghost-like cells with cell viability that was markedly impaired.

Regulation of ethanol-induced apoptosis by glutathione

Considering the above results indicating that oxidative stress levels did not correlate with apoptotic cell death, and the known role of glutathione in regulating redox balance, we next measured GSH levels and the activation of caspase-3 in the WIF-B cells following ethanol administration with or without pyrazole pretreatment and the inclusion of BSO. It was determined that ethanol treatment alone did not affect the content of reduced glutathione in the WIF-B cultures while the overexpression of CYP2E1 in pyrazole-treated cells resulted in a modest decline (20%) of GSH (Figure 3A). Also, as expected, the addition of BSO in the culture media significantly depleted GSH levels in the WIF-B cells (Figure 3A). Strikingly, this depletion of glutathione by BSO resulted in an abrogation of ethanol-mediated

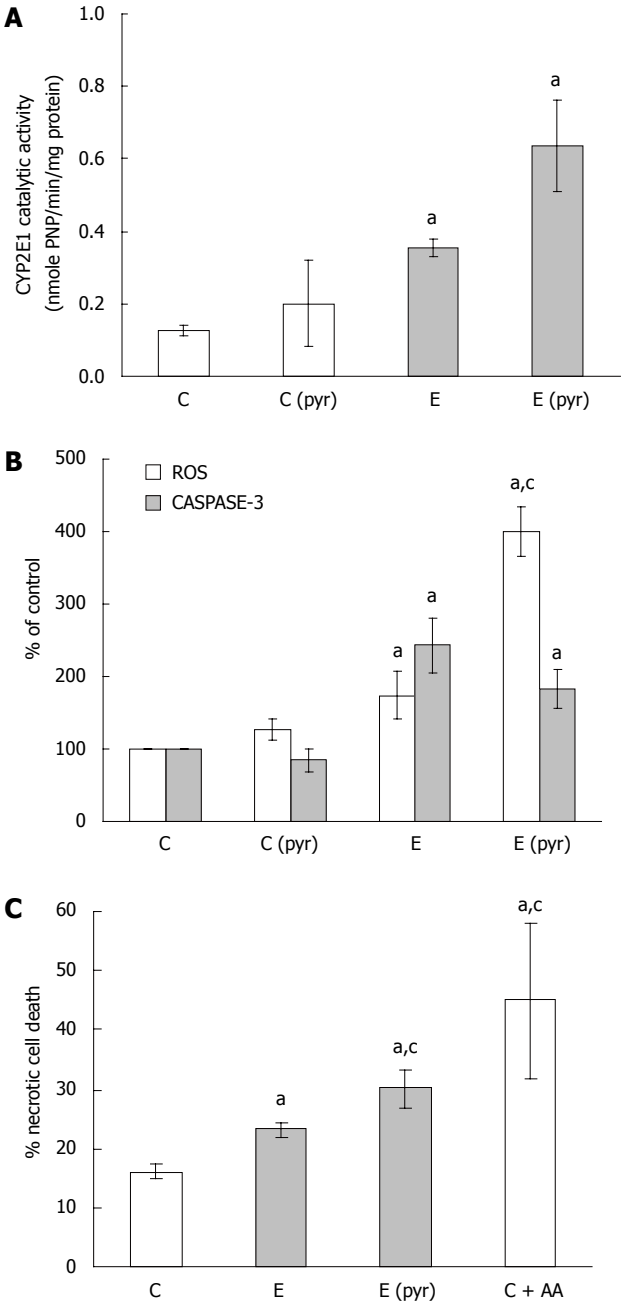

Figure 2 The relationship of CYP2E1 expression, oxidative stress, and WIF-B cell death following ethanol administration. WIF-B cultures were treated without (C) or with pyrazole (Cpyr) for 4 d followed by ethanol treatment for 48 h [E and E (pyr)]. A: CYP2E1 activity in isolated microsomes analyzed using the r-nitophenol oxidation assay in four independent experiments; B: H_2O_2 production representative of ROS generation detected by dichlorofluorescein production, and the activation of caspase-3 as a measure of apoptosis from seven independent experiments; C: Necrotic cell death evaluated by flow cytometric analysis in three independent experiments using the LIVE/DEAD cell stain as described in "Material and Methods". $^{a}P < 0.05$ *vs* control; $^{c}P < 0.05$ *vs* ethanol alone-treated cells.

caspase-3 activation (Figure 3B) as well as the reversal of ethanol-induced morphological changes (Figure 3C).

Fas-mediated apoptosis in ethanol treated WIF-B cells: regulation by GSH

Since the depletion of glutathione by BSO resulted in an abrogation of caspase 3 activity, we next analyzed

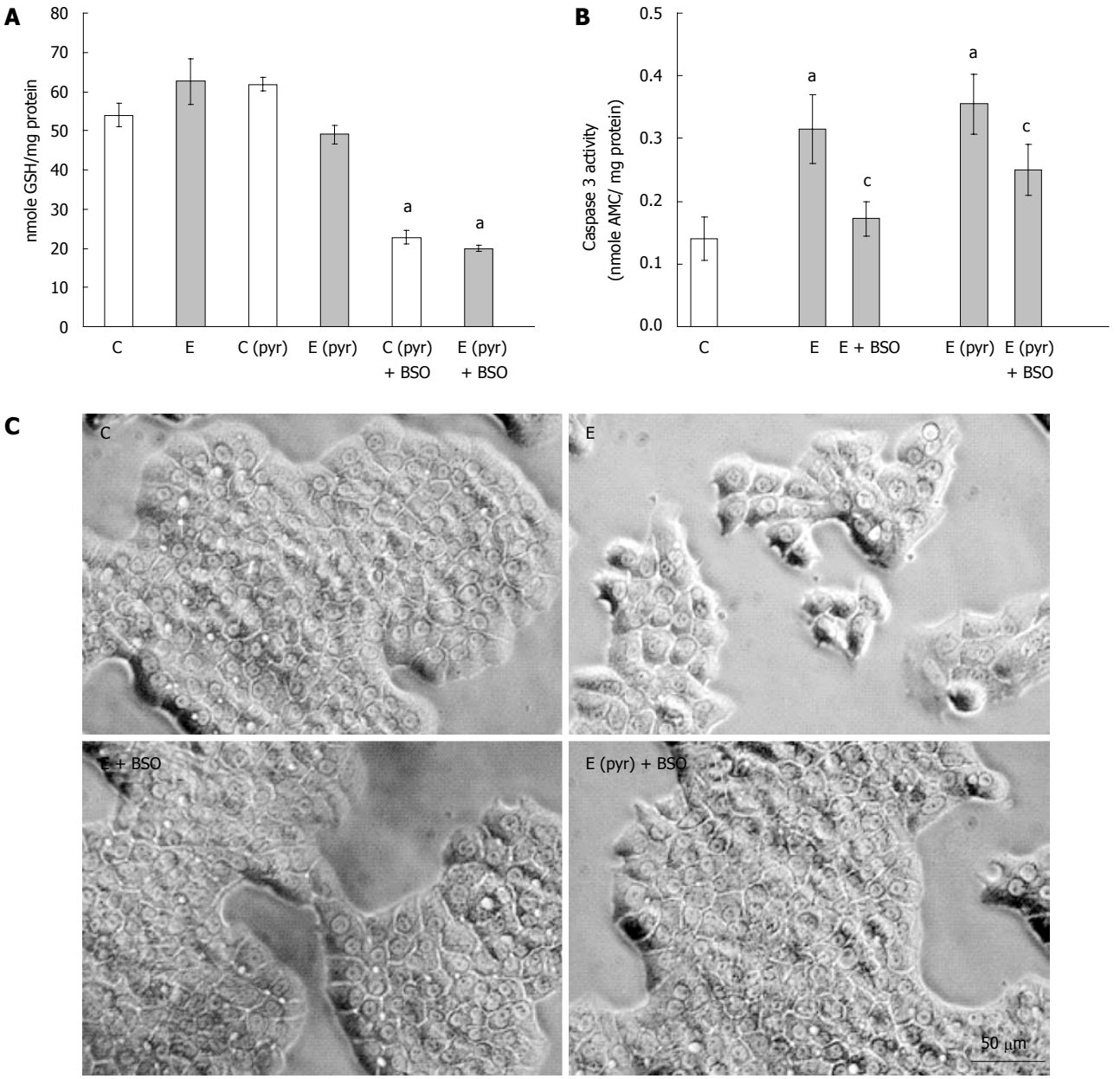

Figure 3 The effect of glutathione depletion on the induction of caspase activation in ethanol-treated WIF-B cells. WIF-B cultures were treated in the presence or absence of ethanol (25 mmol/L) for 48 h after pretreatment with (C pyr and E pyr) or without (C and E) pyrazole. Glutathione was depleted by the inclusion of BSO in the culture media when indicated (+ BSO). A: The amount of reduced glutathione (GSH) was detected as described in "Material and Methods"; B: Measure of apoptosis in WIF-B cells following glutathione depletion. Cell lysates were assayed for caspase-3 activity and the release of fluorescent product was detected and expressed for five independent experiments; C: Phase contrast images of the treated WIF-B cultures. $^{a}P < 0.05$ *vs* control; $^{c}P < 0.05$ *vs* ethanol-treated.

how such events would affect Fas-mediated apoptotic mechanisms which we have previously reported as a potential contributing pathway involved in ethanol-mediated cell death[5]. WIF-B cultures were treated with ethanol for 24 h prior to overnight challenge with anti-Fas antibody/ActD. Following treatment with anti-Fas antibody, both control and ethanol-treated cells greatly increased the Fas-dependent DNA ladder formation (Figure 4A). However, maximal DNA degradation was observed in the cells exposed to ethanol prior to Fas antibody challenge (Figure 4A). Similarly, the increase in caspase-3 activity observed after ethanol treatment alone was found to be further exacerbated when cells exposed to alcohol were treated with Fas antibody (Figure 4B). And finally, when the activation of caspase-8 was measured, the inclusion of BSO in the media was found to significantly reduce the activation of this upstream initiator protease which is linked to Fas-mediated pro-apoptotic events (Figure 4C).

DISCUSSION

There is ample evidence indicating that ethanol administration results in hepatocellular apoptosis, yet a comprehensive understanding of contributing mechanisms remains incomplete. Previously, we have shown that

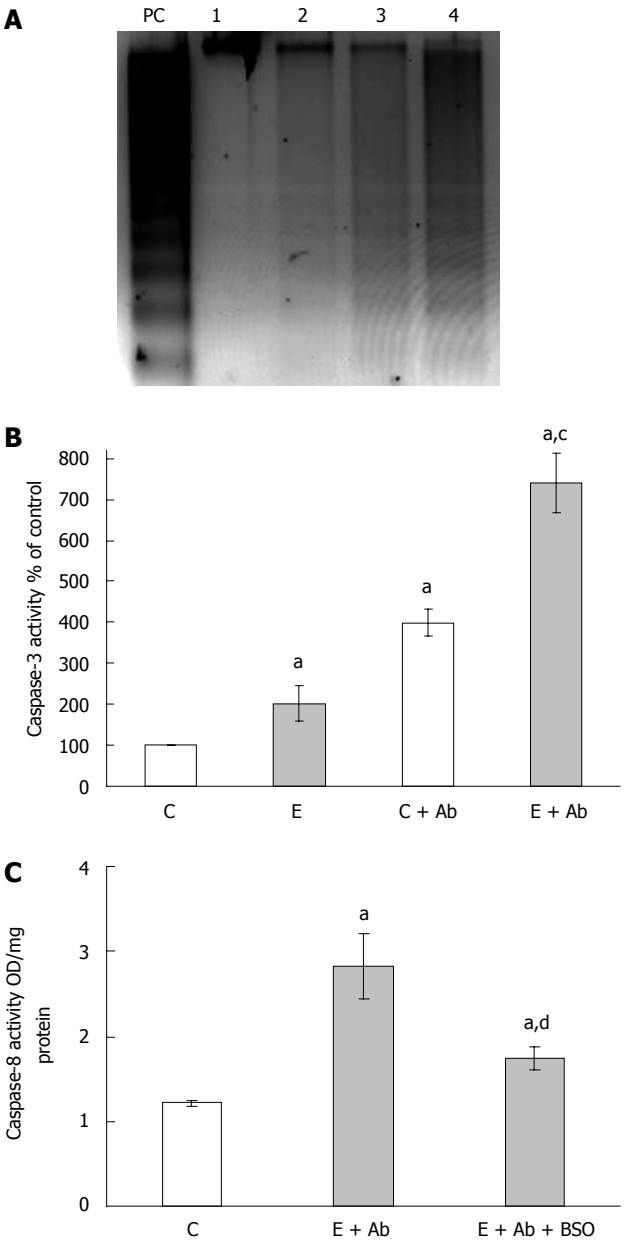

Figure 4 Fas-mediated apoptosis following ethanol administration and the effects of BSO treatment. A: Representative gel depicting apoptosis identified by DNA fragmentation. WIF-B cultures were left untreated or treated with 25 mmol/L ethanol for 24 h prior to challenge with anti-Fas antibody/Act D (Ab) for 16 h. The samples represented are untreated control cells (lane 1), ethanol alone-treated cells (lane 2), control cells incubated with Ab (lane 3), and ethanol-treated cells incubated with Ab (lane 4). UV-treated WIF-B cells were used as a positive control (lane PC); B: The analysis of caspase-3 activation following Fas antibody treatments. WIF-B cells were cultured for 24 h in the absence and presence of ethanol followed by the additional overnight incubation with Ab as described above. The activity of caspase-3 was assayed in four independent experiments as previously described; C: The effect of glutathione depletion by BSO on caspase-8 activation. WIF-B cultures were treated with ethanol and Fas antibody with or without the inclusion of BSO. The activity of caspase-8 was determined in three independent experiments as described in "Material and Methods". $^{a}P < 0.05$ *vs* untreated control cells; $^{c}P < 0.05$ *vs* corresponding controls; $^{d}P < 0.05$ *vs* cells treated with ethanol plus Ab.

ethanol induces apoptosis in WIF-B cells partly as a consequence of ADH-mediated ethanol metabolism, and was associated with death receptor-mediated events, particularly the membrane localization of Fas and the subsequent activation of caspases[5]. In the present study, we have corroborated the role of death-receptor triggers as ethanol-treated WIF-B cells were found to be sensitive to Fas-mediated apoptosis. Additionally, we further defined that ethanol-mediated apoptosis of the WIF-B cells requires an adequate cellular level of glutathione, and that increases in ROS generation are not necessarily associated with the promotion of proapoptotic pathways.

The generation of oxidative stress and mitochondrial related alterations have been implicated as a key mechanism in various pathological systems, including ethanol-mediated hepatocellular damage. Particularly relevant to ALD is the fact that the liver expresses an ethanol-inducible form of one of the cytochrome P450 isoforms (CYP2E1), that is involved in the generation of ROS[7], which may contribute to hepatocellular injury. In the current work, we demonstrated that ethanol treatment of WIF-B cells increases ROS and lipid peroxidation products and that this could be prevented by the presence of DAS or antioxidants, confirming the role CYP2E1 plays in ethanol-mediated oxidative stress in hepatocytes. However, protection from apoptotic cell death was not observed when CYP2E1 was inhibited or when antioxidants were present. To further clarify these observations, the expression of CYP2E1 was manipulated in the WIF-B cells by pyrazole pre-treatment prior to ethanol administration. The results of those experiments demonstrated that the content and activity of CYP2E1 could be increased and that the enhanced induction was correlative to an observed increase of ROS detected in the cells. However, the CYP2E1-related enhancement of oxidative stress in the WIF-B cells did not correlate with an increase in apoptotic cell injury, but rather to necrosis. These results support the hypothesis that when oxidative stress is enhanced, a concomitant decrease in viability and caspase activation occurs that changes the mode of hepatocellular death from apoptosis to necrosis.

Also associated with oxidative stress mechanisms and the adverse pathology present in ALD are alterations that occur to the antioxidant defense mechanisms within the liver. In particular, a vital defense mechanism against oxidative stress is the tripeptide glutathione, the cellular level of which has been implicated as a critical factor in whether a cell survives or succumbs to death mechanisms induced by toxins. However, the effect of ethanol on GSH levels as well as the role of glutathione in apoptosis remains controversial as ethanol administration has been reported to change hepatic GSH levels in both positive and negative ways[9-11]. Differences between studies included the mode and amount of ethanol administered, as well as the animal or cell culture system utilized. The present study represents a hepatic cell culture model (WIF-B cells) that adequately mimics *in vivo* hepatocyte functions, a trait that is sought for model systems for accurate comparisons to human pathology. Specifically, WIF-B cells efficiently metabolize ethanol and express signs of ethanol-mediated damage while using physiologically relevant levels of alcohol (25 and 50 mmol/L). This is often in

contrast to other *in vitro* models that utilize upwards of 100 mmol/L ethanol treatments. In using the WIF-B cell model, we have demonstrated in this study that GSH levels were found to be unaltered by ethanol treatment alone. Additionally, a decline in GSH levels in ethanol-treated cells was observed only after intracellular oxidation was increased due to enhanced activation of CYP2E1 following pyrazole pretreatment. Furthermore, inclusion of the glutathione synthesis inhibitor BSO resulted in the suppression of caspase activation, thereby providing protection against ethanol-induced apoptosis. A potential contributing factor that may be involved in this observed GSH-mediated protection from apoptotic cell death is the fact that caspase-8 activity was found to be diminished when GSH was depleted. This observation is supported by other works demonstrating that sustained glutathione levels may be required for caspase-dependent apoptosis because of the redox-sensitive nature of the cysteine proteases[12,15]. Also, the mode and extent of glutathione removal used in this study may be an important factor as it has been noted that acute GSH depletion can result in the inhibition of Fas-mediated apoptosis, whereas prolonged GSH depletion can override protective anti-apoptotic actions and thus mediate an enhancement of apoptotic cell death[16].

In summary, the liver has been shown to be highly susceptible to Fas-mediated injury and this study has demonstrated that ethanol administration to WIF-B cells can trigger signals associated with this pathway as well as induce susceptibility to this form of apoptotic cell damage. Also, this work has determined that apoptosis induced as a consequence of ethanol metabolism is not completely dependent upon ROS status but is dependent on sustained GSH levels. Thus, factors that regulate apoptotic/necrotic cell death mechanisms and signal the selection of one over the other during ethanol treatment are in part related to the concentration of ROS generated as well as to the ability of antioxidant defenses to cope with the elevated oxidative stress. Overall, the data presented here support the hypothesis that hepatocellular damage which occurs during the early stages of alcohol-mediated injury (i.e. steatosis) may involve the preferential signaling of apoptotic mechanisms whereas stages of more advanced disease (e.g. steatohepatitis and fibrosis) may involve more necrotic rather than apoptotic cell death of hepatocytes as the liver's sensitivity to oxidative stress mechanisms is enhanced.

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COMMENTS

Background

It has been documented that the clinical progression of alcoholic liver disease is associated with an increase in hepatocellular damage which may involve the promotion and execution of apoptotic death mechanisms. It has also been shown that as a consequence of ethanol metabolism, oxidative stress is induced in hepatocytes through the generation of reactive oxygen species (ROS), such as hydrogen peroxide and superoxide. These oxidants can promote hepatotoxicity by inducing protein oxidation, enzyme inactivation, lipid peroxidation, and the production of reactive aldehydes. However, the relationship between hepatocellular oxidative stress and the promotion of apoptotic cell injury is not completely understood. This study is part of current efforts aimed at clarifying pathways and mechanisms associated with ethanol-mediated hepatotoxic events.

Research frontiers

As part of the effort to dissect parameters that are involved in ethanol-related signaling and its adverse effects, researchers have utilized a variety of models. This study utilizes hepatic hybrid WIF-B cells as an *in vitro* model for the study of alcohol-associated hepatocellular alterations. This is an emerging model for studying the effects of ethanol on cellular processes that is showing immense promise as these cells endogenously express ethanol metabolizing enzymes (alcohol dehydrogenase and CYP2E1), and as a consequence, have been shown to exhibit classic alcohol-mediated adverse effects such as triglyceride accumulation. In this work, the use of the WIF-B cells has brought forth new information concerning the relationship of oxidative stress and cell death following ethanol treatment in a model that provides a more accurate comparison to human pathology than other culture systems.

Innovations and breakthroughs

This study demonstrates that ethanol administration not only results in the trigger of signals associated with the Fas death receptor pathway, but that ethanol also primes hepatocytes, making them more susceptible to apoptotic damage. Also, we showed that apoptosis induced as a consequence of ethanol metabolism in the hepatoma cultures was not completely dependent upon oxidative stress mechanisms and was related to sustained cellular glutathione levels. Thus, this work implies that the status of thiol levels in hepatocytes may predict what hepatotoxic signaling events (i.e. apoptotic or necrotic) are triggered by the corresponding level of ethanol exposure and oxidative stress.

Applications

The prevalence and progression of alcohol-induced liver disease is a major health concern worldwide. Many prior studies have shown that the enhancement of adverse outcomes and pathological damage is associated with several parameters, including the induction of oxidative stress and the promotion of hepatocellular death mechanisms. This study provides evidence indicating that as oxidative stress in hepatocytes is enhanced (a condition related to increased ethanol consumption and/or duration of use); a change in the mode of cell injury occurs from mechanisms that support proapoptotic events to those involved in passive necrosis of the cell. This information may aid in the development of therapeutic interventions for use at appropriate stages of the disease process.

Terminology

WIF-B cells: Highly differentiated cells that are a clone of a human fibroblast (WI 38) and a Fao rat hepatoma cell. These cells develop a hepatocellular-polarized phenotype in culture and efficiently metabolize ethanol.

Peer review

This study is a continuation of a series of studies reported by this group. In this extended study they have proved that ethanol induced enhanced susceptibility of Fas mediated apoptosis of polarized hepatic cells (WIF-B) is dependent on sustained glutathione levels and only partially dependent on ROS status.

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Effective use of FibroTest to generate decision trees in hepatitis C

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Abstract

AIM: To assess the usefulness of FibroTest to forecast scores by constructing decision trees in patients with chronic hepatitis C.

METHODS: We used the C4.5 classification algorithm to construct decision trees with data from 261 patients with chronic hepatitis C without a liver biopsy. The FibroTest attributes of age, gender, bilirubin, apolipoprotein, haptoglobin, α2 macroglobulin, and γ-glutamyl transpeptidase were used as predictors, and the FibroTest score as the target. For testing, a 10-fold cross validation was used.

RESULTS: The overall classification error was 14.9% (accuracy 85.1%). FibroTest's cases with true scores of F0 and F4 were classified with very high accuracy (18/20 for F0, 9/9 for F0-1 and 92/96 for F4) and the largest confusion centered on F3. The algorithm produced a set of compound rules out of the ten classification trees and was used to classify the 261 patients. The rules for the classification of patients in F0 and F4 were effective in more than 75% of the cases in which they were tested.

CONCLUSION: The recognition of clinical subgroups should help to enhance our ability to assess differences in fibrosis scores in clinical studies and improve our understanding of fibrosis progression.

Key words: Hepatitis C; FibroTest; Decision trees; C4.5 algorithm; Non-invasive biomarkers

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INTRODUCTION

One of the most widely used non-invasive markers to stage liver fibrosis is the FibroTest (FT; BioPredictive, Paris, France), which involves the measurement of surrogate markers, α2 macroglobulin (A2M), haptoglobin, γ-glutamyl transpeptidase (γGT), total bilirubin, and apolipoprotein A1 (APO-A1), which,

in combination, have a high predictive value for the diagnosis of significant fibrosis[1-5]. The correlation of these markers with liver fibrosis involves a formula that was derived through logistic regression using data from 339 patients. The data were also used for the construction of a classifier using neural networks, but logistic regression was favored[1]. Its efficacy has been further validated by comparing the predictions made by the formula to those obtained with histological scoring of liver biopsies[6-10].

FT is employed to evaluate a complex situation under conditions of uncertainty, e.g. evaluating the degree of fibrosis in a patient with hepatitis C. A recent review of FT performance in 6549 patients and 925 controls supported the recommendation in clinical practice of FT as an alternative to liver biopsy for the first-line assessment of liver injury in patients with chronic hepatitis C. This review concludes that neither biomarkers nor biopsies are sufficient alone to provide the information necessary to make definitive decisions in a given patient, but rather, all the clinical and biological data must be taken into account[11].

Based on experience obtained, acquiring new information about the behavior of FT in clinical practice will be useful to assess changes to the evaluation of patients with liver fibrosis. In addition, there is a rich set of automatic classification techniques developed within the context of machine learning using Artificial Intelligence, which can be used to simplify the classification process and to provide additional information to support the classification rational. One such technique is the automatic generation of decision trees. Decision trees provide explicit rules to relate the range of values of the biomarkers with fibrosis scores, and they might help to gain a better grasp of the importance and significance of the test.

MATERIALS AND METHODS

Patients

A total of 261 patients with chronic hepatitis C, who were HCV RNA+, not receiving any antiviral or antifibrotic treatment, in whom a liver biopsy could not be obtained and who had been submitted to the FibroTest as part of their first evaluation profile, were included in the study. The patients were recruited from the liver unit of Clinica Lomas Altas, Mexico City from January 2003 to December 2007.

For each patient, we retrospectively gathered data on age, gender, γGT, ALT, AST, total bilirubin, hemoglobin, white cell counts and platelets. All the analytical studies were performed independently of the present study and their results had been reported previously. The interval between routine blood test and FT was less than 5 d.

FibroTest

The FibroTests were performed according to published recommendations. This method provides a quantitative estimate of liver fibrosis ranging from 0.00 to 1.00. The FT cutoffs for presumed fibrosis stages were 0.00-0.21 (F0), 0.22-0.27 (F0-F1), 0.28-0.31 (F1), 0.32-0.48 (F1-F2), 0.49-0.58 (F2), 0.59-0.72 (F3), 0.73-0.74 (F3-F4) and > 0.75 (F4)[12]. Each attribute (component) of the FibroTest was considered and included in the construction of decision trees.

Decision trees

Decision trees are a diagrammatic representation of a decision process, where nodes represent questions about attribute values or ranges of values, and edges represent the possible answers that link question nodes with other nodes down the tree, which represent further questions. Nodes at the bottom of the tree represent classes: the class of an object satisfying all the questions associated to the nodes in the path from the top question node to the bottom class node. In the case of FibroTest, each question node in the tree represents an FT biomarker (e.g. bilirubin and apolipoprotein A1) value or value interval, and the bottom nodes represent the FT scores (i.e. F0, F1, F1-F2, *etc*). In the present study, decision trees were constructed using the C4.5 classification algorithm[13,14]. The C4.5 Algorithm, often referred to as statistical classifier, is based on the concepts of information entropy and information gain. Intuitively, information entropy is the number of bits required to code an event (i.e. a random variable), where the higher the probability of the event the lower the number of bits required to code it. Information gain, in turn, is the reduction of entropy when additional information is available. C4.5 uses the fact that each attribute of data can be used to make a decision that splits the data into smaller subsets, which have reduced information entropy. The C4.5 algorithm is freely available, and for the purpose of this study we used the code supplied directly by Quinlan at http://www.rulequest.com/Personal/.

The simplified pseudo-code for the algorithm is as follows: (1) Find the most informative attribute (i.e. the one with the lowest entropy or the largest information gain) in relation to the set of samples provided. (2) Create a decision node that splits on the selected attribute; this node will have a decision question on an attribute's value or value interval, and will partition the samples in relation to such a value (yes/no) or value interval (i.e. the ones with a lower value, the ones in range, and the ones with a higher value.) If all the samples belong to the same partition, the corresponding node becomes a class node. (3) Create a daughter node for each remaining case, and apply the procedure from (1) for all samples that remain in the corresponding partition.

For the experiments, the following predictive attributes were used: age, gender, bilirubin, Apo A1, A2M, GGT, and haptoglobin. The target was the FibroTest score. The classifier was built with data from the 261 patients. The algorithm also selects the best rules (i.e. paths) from the trees, and makes a set of compound rules, which are tested against all samples in the test-data, and provide a confidence factor. In the present study we tested the classification performance with these compound rules and computed the corresponding confusion matrix.

In order to enhance the confidence of the classifier, we used all the data for training and also for testing

Table 1 Clinical and laboratory data of the study population

Patients with chronic hepatitis C (n = 261)	Mean (range/SD)
Age (yr)	52 (20-78)
Age at infection (yr)	26.74 (birth-69)
Time of progression (yr)	26.41 (2-69)
Hemoglobin (g/dL)	14.83 ± 2.54
Platelet count (10^3/mm^3)	203 ± 82
White cell count (10^3/mm^3)	5.39 ± 1.82
γGT (IU/L)	77 ± 81
ALT (IU/L)	96.4 ± 108.1
Bilirubin (mg/dL)	0.9 ± 0.6

Table 2 Fibrosis stage (FibroTest) distribution according to gender in the study population n (%)

FibroTest score	Female	Male	Total
F0	16	4	20 (8)
F0-F1	5	4	9 (3)
F1	5	4	9 (3)
F1-F2	29	18	47 (18)
F2	21	13	34 (13)
F3	26	15	41 (16)
F3-F4	3	2	5 (2)
F4	44	52	96 (37)
Total	149 (57)	112 (43)	261 (100)

through 10-fold cross-validation as follows: Partition the whole set of available empirical data in 10 randomly generated designated equal subsets; use 90% as train-data and the remaining designated 10% as test-data and compute the classifier's performance. The procedure is repeated using the remaining designated 10% partitions as test-data. The performance of the classifier was presented as the average of the 10 tests.

RESULTS

Demographic data

Of the 261 patients, there were 149 (57%) female and 112 (43%) male. Their mean age was 52 years (range 20-78 years). The mean age at infection was 26 years (range birth to 69 years). 75.1% were genotype 1. The average time from exposure to risk factor to their first FibroTest was 26.4 years. The mean values for the following parameters was as follows: Hb 14.8 ± 2.5 g/dL, platelets 203 000 ± 82 000 10^3/mm^3, leukocytes 5399 ± 1821 10^3/mm^3, serum bilirubin 0.9 ± 0.67 mg/dL, ALT 96.4 ± 108 IU/L, and GGT 77.5 ± 81.0 IU/L (Table 1).

FibroTest

The reported FT scores indicate that 45% of the patients (n = 117) had either F4 (37%) or F0 (8%). The remaining 55% (n = 144) had intermediate stages of fibrosis (Table 2).

Decision trees

The C4.5 algorithm was used to construct ten decision trees. The algorithm selected a number of rules relating attribute values with the fibrosis score and the percentage of times that each rule was successfully applied for each tree. In addition, the algorithm produced a set of 26 compound rules out of the ten classification trees and these rules were used to classify the 261 patients. The plausible rules for the classification of patients in F0 and F4 are shown in Table 3.

Table 3 Compound rules generated for classes F0 and F4 and the percentage of times that each rule was successfully applied

F0	F4
GGT ⩽ 108 IU/L	Bilirubin > 1.2 mg/dL
A2M ⩽ 280 g/L	GGT > 26 IU/L
Apo A1 > 144 g/L	A2M > 216 g/L
Age > 36 yr	Class F4 (95.6%)
Age ⩽ 53 yr	
Class F0 (79.4%)	
	A2M > 335 g/L
	Haptoglobin ⩽ 54.6 g/L
	Age > 53 yr
	Class F4 (95.5%)
Bilirubin ⩽ 1.1 mg/dL	
A2M ⩽ 243 g/L	
Apo A1 > 126 g/L	
Haptoglobin > 73.5 g/L	Gender = M
Age ⩽ 50 yr	Bilirubin > 0.5 mg/dL
Class F0 (77.7%)	A2M > 396 g/L
	Class F4 (91.7%)
	Bilirubin > 0.6 mg/dL
	Haptoglobin ⩽ 16.2 g/L
	Class F4 (90.9%)
	GGT > 40 IU/L
	A2M > 372 g/L
	Age > 59 yr
	Class F4 (90.2%)

Table 4 Confusion matrix relating real FT scores (rows) to predicted FT scores (columns) in 261 patients with chronic hepatitis C

	F0	F0-F1	F1	F1-F2	F2	F3	F3-F4	F4
F0	18					2		
F0-F1		9						
F1			7			2		
F1-F2	2			37	1	7		
F2				3	18	13		
F3						38		3
F3-F4						1	3	1
F4			1			3		92

FT cases with true scores of F0 and F4 were classified with very high accuracy (18/20 for F0 and 92/96 for F4), which indicated that in the extreme stages of fibrosis, decision trees produced the correct classification in approximately 93% of the cases, as shown in the confusion matrix in Table 4.

The overall classification error was 14.9% (accuracy 85.1%). We observed that the largest confusion relates to false positives in F3 (43.7%). However, the chances that the predicted value is right for all FT scores, except F3, are quite high.

DISCUSSION

The FT scores used to evaluate the degree of liver fibrosis were generated using a formula obtained

through logical regression that can be thought of as a classification device or classifier. From this perspective the FT formula is a black box that has the FT attributes as inputs and produces the corresponding FT score as its associated output. However, it would be convenient to be able to look into the internal classification process and have access to the classification rational. In addition, it is also important to reinforce the reliability of the FibroTest and ensure that the classification results are independent of contingent features of the classification technique. Indeed, in the original formulation of the FibroTest, the use of neural networks, another black box classification technique, was also explored but logistic regression was preferred for clarity[1].

As with all machine learning techniques, decision trees such as those employed in the present work, are deduced from empirical data and the success of a given application depends on the quantity and quality of these data. In this respect, these algorithms are analogous to statistical regression techniques, such as logical regression, but rely on classification heuristics. They have proved to behave well not only in linear problems, but also in non-linear or unstable domains. For this, classifiers need to be built with a portion of the data, which is usually called the "train-data", and tested with a different portion of the data, which is usually called the "test-data", and it is essential that these two sets are distinct. For the induction process proper, the values of the attributes of each sample, "the predictors", are associated with its corresponding class, "the target", and the process is repeated iteratively for all the samples in the train-data. At the end of this process, each class is associated with a combination of values or value intervals of the attributes. The classifier can then be used to predict the class of a sample not used in the training process. In particular the performance of the algorithm can be assessed by comparing the known class of each sample in the test-data with the class predicted by the decision tree for such sample. The specifics of these procedures, with the heuristics employed, give rise to a large variety of classification techniques, one of which is decision trees. Decision trees can be created through a diversity of algorithms and the field as a whole is quite mature and has been applied to a large diversity of application domains with very positive results. In particular, in the clinical setting, decision trees have been applied, for instance, to proteomic data analysis in pancreatic cancer[15], to the prediction of interferon treatment effects based on microarray gene expression profiles[16], and to the prediction of diagnosis and outcome of dengue fever based on clinical, hematological and virological data[17].

Ranking the seven attributes used by the FT, we generated a learning set that allowed for the determination of a second classifier using an ensemble of decision trees. To identify the decision algorithms, we used the C4.5 decision tree classifier, which has several advantages over other statistical tools. Indeed, decision algorithms so generated are simple to understand, and they are able to handle missing values. In contrast, logistic regression and discriminant analyses require much more data preparation and more extensive handling of missing values for reliable calculations[18]. Decision algorithms are also easy to interpret and validate using common statistical techniques, which facilitates their use to predict the diagnosis and prognosis in different clinical settings.

The decision tree analysis of our data produced a set of seven plausible rules that correctly predicted the fibrosis score in more than 75% of the cases for F0 and F4. Interestingly, the rules for predicting F4 were precise in 90% of the cases. Therefore, the rules generated herein can be considered as having great accuracy. These findings add support to the impression that fibrosis at the extremes of the disease is more predictable, a notion that applies both to non-invasive markers and liver biopsy[4,19].

Of the markers employed in the decision trees and the derived rules, the most relevant were age and α-2 macroglobulin as independent predictors. If a patient has an FT score of F0 with a normal A2M and is below 53 years of age, our results suggest the presence of mild disease, adding to the clinical decision of not performing a liver biopsy and holding back the timing and selection of antiviral treatment. On the other hand, if a patient with hepatitis C and FT score of F4 is above 53 years and has an increased A2M, a diagnosis of significant fibrosis is presumed.

Fibrosis progression tends to vary between patients and even in the same person, for reasons that are not yet understood. However, age has consistently been reported as an important risk factor for fibrosis progression in chronic viral hepatitis, either at the onset of the disease or during its evolution. The changes with age tend mostly to be subtle and are consistent with a disease of long duration associated with the progression of normal aging[20-23]. How and why variants arise probably relates to changes in extracellular matrix[24,25], liver regeneration[26] and repair mechanisms[27]. In this regard, and based on our results, A2M is an extremely useful attribute. It is a protease inhibitor and a major carrier of cytokines synthesized by hepatic stellate cells and hepatocytes. Furthermore, its expression might inhibit matrix remodeling during fibrosis[28,29].

Neither age nor A2M alone has been proven to be an adequate marker of fibrosis, which makes it important to apply a more comprehensive approach in the use of non-invasive markers for liver fibrosis. Undoubtedly, not knowing which markers are the most predictive has been one of the main obstacles impeding their integration into clinical practice and patients' management. Our study indicates that the combination of the markers used in the FT is reliable and performs well, independently of, age or gender. Although ethnicity was not an inclusion criteria for our study, all the patients were Hispanics with an age range of 20-78 years.

There are pitfalls and caveats to FT use, and the decision tree analysis was not able to generate accurate rules to predict intermediate FT scores (particularly F3), suggesting that the FT attributes for these particular

stages exhibit considerable noise or are inconclusive. This was reflected in the analysis of the confusion matrix in which F3 was the most ambiguous; nonetheless, our study showed that restricting the biopsies to the patients with intermediate scores (F1-F3) could have prevented liver biopsies in 42% of the patients while maintaining an accuracy level above 75%. This is a strong argument to include the use of a non-invasive marker of fibrosis, such as the FT, in the profile evaluation of a patient with chronic hepatitis C, if for any reason; a liver biopsy cannot be performed.

Outside of clinical trials, with the advancement of new laboratory techniques, such as PCR and non-invasive biomarkers, more patients are treated for chronic fibrotic liver diseases without a liver biopsy[30], which remains the best predictor but is not necessarily the gold standard.

Analysis, such as the one performed in the present work, could help to further classify preclinical subgroups and identify subclasses of rapid or slower fibrosers. This classification should enhance our ability to assess differences in fibrosis scores in clinical studies and improve our understanding of fibrosis progression.

This work was presented as a poster at the AASLD Liver Meeting in November 2008 in San Francisco, California.

COMMENTS

Background

One of the most widely used non-invasive markers to stage liver fibrosis is the FibroTest which involves the measurement of a set of surrogate markers that, in combination, have a high predictive value for the diagnosis of significant fibrosis.

Research frontiers

The FT score is obtained with a formula generated through logical regression that can be thought of as a classification device or classifier. It would be convenient to be able to look into the internal classification process and have access to the classification rational. In addition, it is also important to reinforce the reliability of the FibroTest and ensure that the classification results are independent of contingent features of the classification technique.

Innovations and breakthroughs

The authors used the automatic generation of decision trees to simplify the classification process and to provide additional information to support the classification rational of the FibroTest as a non-invasive marker of liver fibrosis. Decision trees provide explicit rules to relate the range of values of the biomarkers with fibrosis scores, and they might help in gaining a better grasp of the importance and significance of the test.

Applications

Analysis, such as the one performed in the present work, could help to further classify preclinical subgroups and identify subclasses of rapid or slower fibrosers. This classification should enhance our ability to assess differences in fibrosis scores in clinical studies and improve our understanding of fibrosis progression.

Terminology

Decision trees are diagrammatical representations of decision processes, where nodes represent questions about attribute values or range of values, and edges represent the possible answers that link question nodes with other nodes down the tree, which represent further questions. Nodes at the bottom of the tree represent classes: the class of an object satisfying all the questions associated to the nodes in the path from the top question node to the bottom class node.

Peer review

This is a timely, well written article on FibroTest.

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Long-term results of endoscopic balloon dilatation of lower gastrointestinal tract strictures in Crohn's disease: A prospective study

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Abstract

AIM: To examine the long-term results of endoscopic treatment in a prospective study conducted over a period of 10 years, 1997 to January 2007.

METHODS: A total of 25 patients (20 female and five male: aged 18-75 years), with at least one symptom of stricture not passable with the standard colonoscope and with a confirmed scarred Crohn's stricture of the lower gastrointestinal tract, were included in the study. The main symptom was abdominal pain. The endoscopic balloon dilatation was performed with an 18 mm balloon under endoscopic and radiological control.

RESULTS: Eleven strictures were located in the colon, 13 at the anastomosis after ileocecal resection, three at the Bauhin valve and four in the ileum. Four patients had two strictures and one patient had three strictures. Of the 31 strictures, in 30 was balloon dilatation successful in a single endoscopic session, so that eventually the strictures could be passed easily with the standard colonoscope. In one patient with a long stricture of the ileum involving the Bauhin valve and an additional stricture of the ileum which were 15 cm apart, sufficient dilatation was not possible. This patient therefore required surgery. Improvement of abdominal symptoms was achieved in all cases which had technically successful balloon dilatation, although in one case perforation occurred after dilatation of a recurrent stricture. Available follow-up was in the range of 54-118 mo (mean of 81 mo). The relapse rate over this period was 46%, but 64% of relapsing strictures could be successfully dilated again. Only in four patients was surgery required during this follow-up period.

CONCLUSION: We conclude from these initial results that endoscopic balloon dilatation, especially for short strictures in Crohn's disease, can be performed with reliable success. Perforation is a rare complication. It is our opinion that in the long-term, the relapse rate is probably higher than after surgery, but usually a second endoscopic treatment can be performed successfully, leading to a considerable success rate of the endoscopic procedure.

Key words: Crohn's disease strictures; Balloon dilatation; Endoscopy; Morbidity; Mortality

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INTRODUCTION

Strictures of the gastrointestinal tract are common complications of Crohn's disease. Medical treatment can improve acute inflammation, but is ineffective in the presence of chronic scarred strictures. These strictures are mainly treated surgically. As well as techniques of surgical resection, especially for less extended strictures, reconstructive surgery (strictureplasty) is

recommended, in order to minimize the risk of short bowel syndrome[1,2]. Strictureplasty is a bowel-conserving operation technique, requiring anesthesia and minimally invasive surgery or laparotomy. Regardless of which surgical technique is chosen, the reoperation rate for new strictures or recurrence of strictures is between 15% and 45% within 5 years[3,4].

For some time, it has been possible to treat benign strictures of the upper gastrointestinal tract with endoscopic balloon dilatation. Over the past 20 years, this method has become increasingly used for symptomatic Crohn's strictures in the ileum or colon[5-16]. The available results indicate equal technical effectiveness of the endoscopic procedures when compared to surgical therapy. Up till now the method has not been standardized, for example regarding balloon diameters or possible concomitant medical treatment.

Valid long-term studies of 5 years or more, which would permit a statement regarding recurrence of strictures and a better comparison with surgical techniques[3,4], are available so far only in casuistic form and with limited numbers[8,17-19].

We report on our own prospective 10-year long-term study of endoscopic balloon dilatation of strictures in Crohn's disease using a relatively thin balloon (18 mm) and additional treatment with prednisolone.

MATERIALS AND METHODS

In our prospective long-term study, conducted since 1997, we included patients who had at least one symptomatic ileal or colonic stricture which could not be passed with the standard colonoscope (external diameter 13 cm), in the presence of histologically documented Crohn's disease. Additional inclusion criteria were: (1) obstructive symptoms (especially abdominal pain), refractory to medical treatment; (2) no or low inflammatory disease activity (CDAI < 200); (3) age over 18 years; (4) no fistulas connecting to the stricture; (5) length of the stenosis no more than 10 cm; (6) patient's consent after being informed about the uncertain success of the method, with regard to current knowledge; (7) Either an enteroclysis (Sellink) or a colon contrast imaging examination was performed to exclude the presence of fistulas and determine the length of the stricture.

Patients were prepared for endoscopy with 4 L of Golytely solution. Premedication consisted of 2.5-5 mg midazolam and 50 mg meperidine. After the dilatation patients were monitored in the hospital for at least 24 h.

The balloon dilatation was carried out through the placed endoscope, under endoscopic and radiological control using a balloon of 55 mm in length and 18 mm in diameter (Olympus BC4). In a safe position within the stricture, the balloon was filled with diluted contrast medium for at least 2 min with a pressure of 2.0 at (= 19 6133 bar).

The dilatation was judged technically successful if the stricture appeared conspicuously larger during radiological control of the balloon diameter, and if it could be passed with the standard colonoscope.

Twenty five patients (20 female, five male) between the age of 18 and 75 years and with a disease duration of between 2 and 35 years (mean 13.3 years) were included in the study. There were 11 strictures located in the colon, 13 strictures at the anastomosis after ileocecal resection, three at the Bauhin's valve and four in the ileum. Four patients had two, one patient had three strictures (Table 1). The length of the strictures was between 1 and 10 cm.

Table 1 Patient characteristics

Case number	Age/sex	Disease duration (yr)	Previous bowel surgery	Stricture localisation
1	38/F	17	Ileocecal resection	Anastomosis
2	22/F	5	None	Transverse colon
3	23/F	7	None	Sigmoid, ileum
4	23/M	7	appendectomy	Ileum
5	33/F	14	None	Rectum/sigmoid
6	33/F	12	Ileocecal resection	Anastomosis
7	32/F	9	Anal fistula	Ileocecal valve
8	46/F	19	Anal fistula	Colon
9	28/F	16	Ileocecal resection, bowel perforation	Anastomosis
10	35/F	15	Ileocecal resection	Anastomosis
11	26/M	7	None	Ileocecal valve
12	42/F	20	Right hemicolectomy anal fistula	Anastomosis
13	48/F	15	None	Colon
14	50/F	10	None	Colon
15	50/F	14	Ileocecal resection	Anastomosis
16	24/F	6	Ileocecal resection	Anastomosis
17	55/F	11	Ileocecal resection, terminal ileum	Anastomosis
18	43/F	25	Ileocecal resection, sigmoid resection	Anastomosis
19	49/F	35	Ileocecal resection, right hemicolectomy	Anastomosis (2)
20	75/M	3	None	Ileum
21	42/M	6	Right hemicolectomy	Anastomosis
22	34/F	13	Anal fissure	Anal channel, rectum, sigmoid
23	26/F	6	None	Rectum, sigmoid
24	18/F	2	None	Ileocecal valve
25	26/M	20	Total colectomy, ileoanal pouch	Pouchanal anastomosis

Regardless of pre-existing medical treatment, after the dilatation all patients received 3 g of peroral mesalamine and initially 50 mg of prednisolone, reducing the dosage gradually over a period of 2 mo.

Our follow-up period was between 54 and 118 mo (mean 81 mo). All patients were last seen in outpatient settings in January 2007 (recent medical history, clinical examination). The study was carried out in accordance with the Helsinki declaration.

RESULTS

In 30 of 31 strictures (24 of 25 patients) balloon dilatation

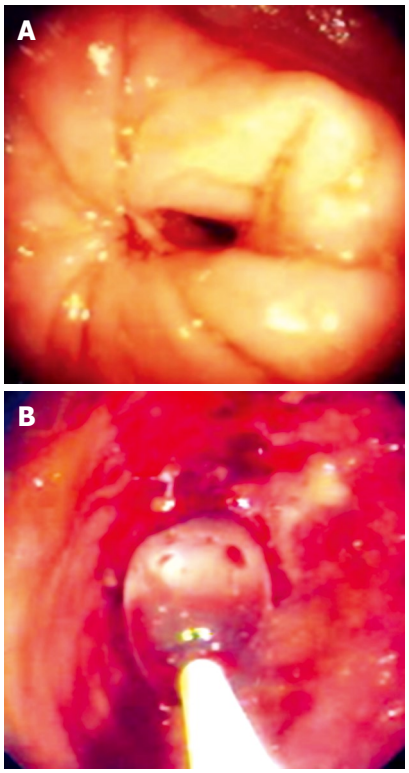

Figure 1 Endoscopic view of a non-passable stricture. A: Ileum; B: An anastomosis, balloon in the stricture.

was successful in one endoscopic session. Eventually the stricture could be passed easily with the standard colonoscope. Only in one patient with a long (about 10 cm) stricture of the ileum, involving the ileocecal valve, and an additional stricture of the ileum 15 cm distant, was the dilatation unsuccessful, so that surgery (ileocecal resection) had to be performed.

With the above mentioned premedication, 10 of 25 patients complained of moderate pain during the dilatation. In one patient with a recurrence of stricture, the second balloon dilatation was complicated by perforation (3%), and consequently the patient had to be operated on. Bleeding requiring treatment or rise of body temperature after dilatation were not observed.

With technical success, the treatment caused immediate improvement of abdominal pain, which was the main symptom in all 24 patients with successful dilatation.

The follow-up period of the successfully dilated patients was between 54 and 118 mo (mean 81 mo). Thirteen of the successfully dilated patients did not have any symptoms indicating a stricture relapse up to the last follow-up in January 2007. In 11 cases, 3 to 77 mo (mean 32 mo) after successful dilatation, a stricture relapse developed which could not be passed with the colonoscope. Accordingly, after a mean follow-up of more than 6.5 years, the relapse rate was 46%. In seven patients with recurrent strictures, a second endoscopic balloon dilatation could be performed successfully, and in only four patients was surgery required (Figures 1 and 2). Thus the long-term success rate of balloon dilatation was 80% over a mean follow-up period of 81 mo.

As accompanying medical treatment all patients received 3 g of peroral mesalamine and initially 50 mg of prednisolone, reducing the dosage gradually over a period of 2 mo. The results of all patients are summarized in Table 2 and Figure 3.

Table 2 Results

Case number	Stricture number	Compli-cations	Follow-up (mo)	Time to relapse (months & method of therapy)
1	1	None	118	77, dilatation
2	1	None	112	No relapse
3	2	None	106	Sigmoid: 10, dilation ileum: no relapse
4	1	None	106	37, operation
5	1	None	101	No relapse
6	1	None	99	30, dilatation
7	1	None	97	Unsuccessful dilatation, operation
8	1	None	97	No relapse
9	1	None	89	No relapse
10	1	None	86	No relapse
11	1	None	84	15, operation
12	1	None	79	No relapse
13	1	None	75	No relapse
14	1	None	75	24, operation
15	1	None	73	55, dilatation
16	1	None	73	40, dilatation
17	1	None	71	48, dilatation
18	1	None	67	No relapse
19	2	None	66	No relapse
20	2	None	65	No relapse
21	1	None	63	14, dilatation
22	3	None	62	No relapse
23	2	None	57	No relapse
24	1	Perforation	54	4, operation
25	1	None	54	No relapse

DISCUSSION

Endoscopic balloon dilatation has been used over a long period of time in the treatment of strictures of the upper gastrointestinal tract, sporadically also for Crohn's strictures of the duodenum[20,21]. Balloon catheters suitable for transendoscopic dilatation of ileal and colonic strictures have made it possible to dilate Crohn's strictures of the lower gastrointestinal tract.

In the studies published so far, balloons with an external diameter of 18-25 mm have been used, in order to enable the endoscopic dilatation of strictures of the lower gastrointestinal tract. The technical success rate, defined as achieving an endoscopically passable residual stricture, is between 70% and 90 %, independent of the balloon's diameter[5-16]. Usually more than one dilatation session is required for every stricture. Complications such as hemorrhages are rare, while perforations are reported mostly in studies in which 25 mm balloons are used[5,8].

In the present study, single-session dilatations using an 18 mm balloon were technically successful in 97% of cases. In one case perforation occurred during attempted dilatation of a relapsing stricture (3%). Ramboer *et al*[12] exclusively used 18 mm balloons in 52 sessions involving 13 patients, without complications. This technique has the additional advantage that the dilatation set can be easily inserted through a standard colonoscope with a

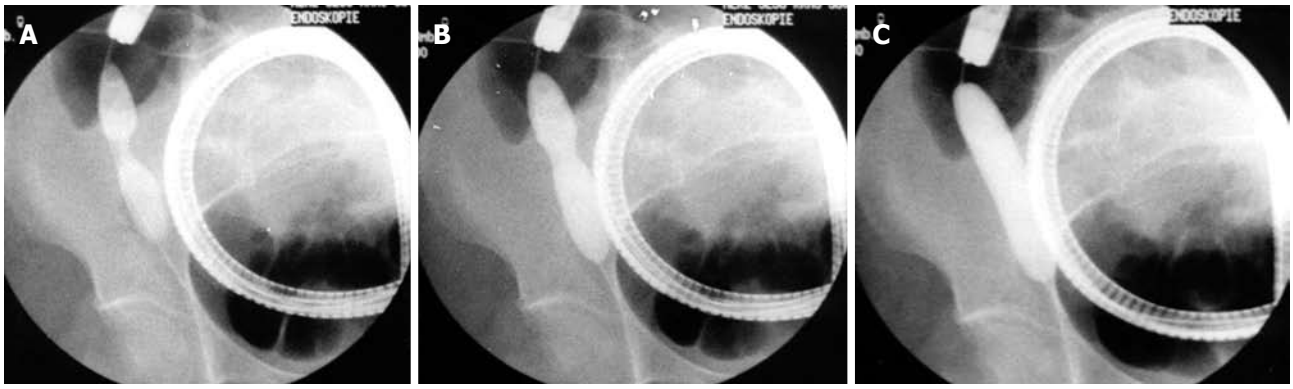

Figure 2 Radiological image of the endoscopic dilatation of a short stricture in the ileum. A: Before dilatation; B: Beginning dilatation; C: Completed dilatation.

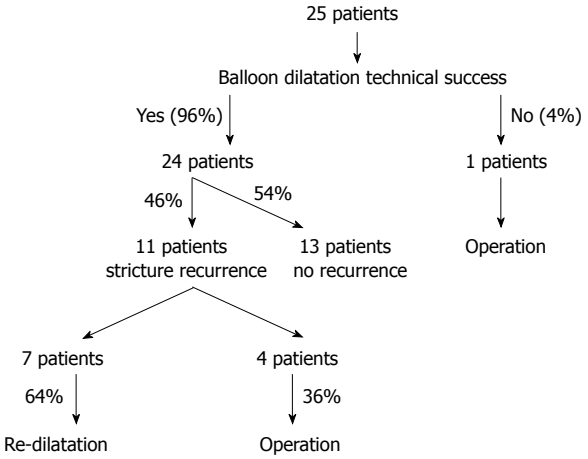

Figure 3 Overview of all patients treated.

working channel of 3.2 mm in diameter. On the other hand, when using balloon catheters with a diameter of 25 mm, therapeutic colonoscopes with a larger working channel are required.

Unfortunately, results of comparative studies on the effectiveness of different balloon diameters and balloon lengths, on differences in duration and pressure of the dilatation, as well as on the number of dilatations per session in the treatment of Crohn's strictures are still lacking. The accomplishment of achieving an endoscopically passable residual stricture seems to be important for the outcome. Couckuyt *et al*[8] found a statistically significant correlation between this achievement and the number of patients being free of symptoms over a longer observation period.

In our experience, intravenous midazolam and meperidine usually are sufficient as accompanying medication. Patients did not complain about a lot of pain during the intervention. Alternatively, in cases of painful procedures, the application of propofol can be considered.

At the moment, it is difficult to define the relapse risk after endoscopic balloon dilatation, as the published studies[8,17-19] are based on very different follow-up periods, and often do not represent structured prospective long-term studies.

In the present prospective long-term study, after a mean follow-up of more than 6.5 years, stricture relapses were observed in 11 patients (46%) after a mean of 32 mo. The other 13 successfully dilated patients were free of symptoms and stricture relapse over the complete follow-up period.

Endoscopic re-dilatation was successful in 64% of the patients with relapsing strictures. The long-term success rate was 80%. Our data indicate that long-term results of endoscopic balloon dilatation, repeated if necessary, are comparable to the results of surgical treatment[3,4]. The relapse rate after a single balloon dilatation is probably higher than after surgical intervention[3,4].

Whether additional medical treatment, such as steroid injection into the stricture, as suggested in case reports[7,11,12], or treatment with steroids after dilatation, can influence the recurrence rate is unknown. Follow-up studies after surgical bowel resection have shown that, after surgical treatment of Crohn's strictures, local inflammatory signs develop rapidly at the anastomosis without clinical signs of a relapse[22].

Raedler *et al*[23] treated 30 patients after successful dilatation of ileocecal strictures with azathioprine 100 mg/d and budenoside 9 mg/d or with a placebo. After 6 and 12 mo, a statistically significantly greater number of symptoms due to strictures and more necessary surgical interventions were found in the placebo group.

In the present study, under hypothetic considerations, a 2-mo steroid treatment was performed after dilatation, aimed at reducing the early relapse rate. With one exception there were no early relapsing strictures in the first 6 mo, and only one repeat dilatation was necessary over this period. Definite conclusions, however, cannot be drawn from these data.

Considering all available results, the endoscopic balloon dilatation seems suitable for the treatment of Crohn's strictures in the lower gastrointestinal tract especially in presence of scarred, short strictures. Our considerable clinical experience has shown us that in cases of multiple strictures, only one stricture should be dilated in each session. Strictures of more than 5 cm in length should instead be treated surgically. Fistulas originating from the stricture are considered contraindications for balloon dilatation.

The relapse rate after single endoscopic balloon

dilatation, according to the results of the present long-term study, is probably somewhat higher than after surgery. But by repeatedly dilating relapsing strictures, surgery often can be avoided or delayed. The long-term success rate of endoscopic dilatation (repeated if necessary) of 80% is remarkable and has to be considered when comparing balloon dilatation with surgery.

COMMENTS

Background

Strictures of the gastrointestinal tract are common complications of Crohn's disease. Medical treatment can improve acute inflammation, but is ineffective in the presence of chronic scarred strictures. These strictures are mainly treated surgically.

Innovations and breakthroughs

The available results indicate equal technical effectiveness of the endoscopic procedures, compared to surgical therapy. This is one of few studies to report prospective 10-year results. Furthermore, our study would suggest that endoscopic balloon dilatation seems suitable in the treatment of Crohn's strictures, especially in presence of scarred, short strictures.

Applications

When considering treatment options for patients with Crohn's disease, this study may show a safe and feasible alternative for therapeutic intervention in the treatment of patients with short, scarred strictures.

Peer review

The authors examined a total of 25 patients with at least one symptom of stricture, not passable with the standard colonoscope and with a scarred Crohn's stricture of the lower gastrointestinal. The study revealed that endoscopic balloon dilatation especially for short strictures in Crohn's disease, can be performed with reliable success. Perforation is a rare complication. The long-term relapse rate may probably be higher than after surgery, but usually a second endoscopic treatment can be performed successfully. The results are interesting and may show a safe and feasible alternative for therapeutic intervention in the treatment of patients with short, scarred strictures.

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BRIEF ARTICLES

Small intestine bacterial overgrowth and irritable bowel syndrome-related symptoms: Experience with Rifaximin

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Abstract

AIM: To estimate the prevalence of small intestinal bacterial overgrowth (SIBO) in our geographical area (Western Sicily, Italy) by means of an observational study, and to gather information on the use of locally active, non-absorbable antibiotics for treatment of SIBO.

METHODS: Our survey included 115 patients fulfilling the Rome Ⅱ criteria for diagnosis of irritable bowel syndrome (IBS); a total of 97 patients accepted to perform a breath test with lactulose (BTLact), and those who had a positive test, received Rifaximin (Normix®, Alfa Wassermann) 1200 mg/d for 7 d; 3 wk after the end of treatment, the BTLact was repeated.

RESULTS: Based on the BTLact results, SIBO was present in about 56% of IBS patients, and it was responsible for some IBS-related symptoms, such as abdominal bloating and discomfort, and diarrhoea. 1-wk treatment with Rifaximin turned the BTLact to negative in about 50% of patients and significantly reduced the symptoms, especially in those patients with an alternated constipation/diarrhoea-variant IBS.

CONCLUSION: SIBO should be always suspected in patients with IBS, and a differential diagnosis is done by means of a "breath test". Rifaximin may represent a valid approach to the treatment of SIBO.

Key words: Rifaximin; Small intestinal bacterial overgrowth; Irritable bowel syndrome; Locally non-absorbable antibiotics

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INTRODUCTION

Irritable bowel syndrome (IBS) is a common gastrointestinal (GI) disease with a prevalence ranging between 11% and 14% of adult population; it is characterized by altered motility, visceral hypersensitivity, abnormal brain-gut interaction, autonomic dysfunction, and immune activation. Although the physiopathological mechanisms underlying IBS are not fully identified, a unifying explanation of symptoms arises from the observation that 92% of IBS patients share the symptom of bloating regardless of their predominant complaint. Thus, the hypothesis has been envisaged that a small intestinal bacterial overgrowth (SIBO) could explain bloating in IBS, based on the following findings: (1) a significantly higher total hydrogen (H_2) excretion after lactulose ingestion in a large percentage (84%) of IBS patients; (2) a 75% improvement of IBS symptoms after eradication of SIBO with locally acting antibiotics; and (3) a tight correlation between the pattern of bowel movement and the type of excreted gas[1].

The prevalence of SIBO among newly diagnosed IBS patients is not exactly known, and the largely variable data reported in literature reflect the different sensitivity and specificity of the methods, either biochemical or microbiological, used to make diagnosis of SIBO. However, an exact estimation of SIBO prevalence should have important therapeutic implications, since SIBO and related symptoms (i.e. abdominal bloating) can be successfully treated by locally active, non-absorbable antibiotics[2,3].

Analysis of breath specimens for volatile metabolites of orally administered substrates offers a simplified detection method for the presence of an abnormal small-intestinal flora; this technique ("breath test", BT) is not only simpler and more acceptable to patients than jejune aspiration,

but also gives quicker information to the clinician than microbiologic culture of the jejune aspirate[4].

The BT with lactulose (BTLact) is based on the properties of lactulose, which is not absorbed through the small intestine and reaches unchanged the colon, where it is metabolized by the bacterial flora. When the bacteria come in contact with lactulose, they metabolize it and produce intestinal gasses, such as methane and hydrogen, which can be detected and assayed in the expired air. In healthy population, bacteria are not present in the duodenum and, therefore, at least 2 h are needed for lactulose to reach the colon and be metabolized. Thus, an increase of hydrogen concentrations in the expired air within 90-120 min strongly suggests a bacterial overgrowth and contamination of the small intestine. The sensitivity and specificity of BTLact in the diagnosis of SIBO are actually estimated to be 68% and 44%, respectively[5]; its main advantage of the BTLact is the relative simplicity of the method, which can be performed by most of the Gastroenterology Units.

By measuring the increased hydrogen concentrations in the expired air after oral lactulose administration (BTLact), an incidence of 46% has been recently found in an Italian survey among 96 patients with IBS[2], which is in agreement with an Europe study demonstrating a significantly increased GI bacterial flora in 43% of IBS patients compared with 12% of matched-control healthy subjects[6], while a USA-based survey has revealed that this incidence may be even higher than 80% of IBS patients[1,7,8].

We, therefore, performed an observational analysis on patients with an initial diagnosis of IBS according to the Rome Ⅱ criteria, in order to estimate the prevalence of SIBO in our geographical area (Western Sicily, Italy) and to gather information on the use of locally active, non-absorbable antibiotics for treatment of concomitant SIBO and IBS.

METERIALS AND METHODS

Our survey included a total of 125 patients who were addressed to our Medical Centre because of abdominal pain and discomfort, in the period ranging between January and December 2006; patients with severe cardiovascular and/or respiratory and/or renal diseases, as well as patients with cancer or under treatment with antibiotics and corticosteroids were excluded.

One hundred and fifteen of these subjects fully complied with Rome Ⅱ criteria, i.e. (1) 3 mo of continuous or recurrent symptoms of abdominal pain or irritation that may be relieved with a bowel movement or coupled with a change in frequency or related to a change in the consistency of stools; (2) two or more of the following symptoms present at least 25% of time: (a) change in stool frequency (> 3 bowel movement daily or < 3 bowel movements weekly); (b) noticeable difference in stool form (hard, loose and watery stools or poorly formed stools); (c) passage of mucous in stools; (d) bloating or feeling of abdominal distention; (e) altered stool passage (e.g. sensations of incomplete evacuation, straining, or urgency).

These 115 subjects received a symptomatological diagnosis of IBS, while the other ten subjects had a diagnosis of either Crohn's disease or ulcerative colitis or celiac disease.

Patients with an IBS diagnosis were asked for their informed consent to the management of personal data, in compliance with the "privacy" regulations in force in Italy. According to their intestinal habits, patients were divided into a constipation-variant (20.6%; six male and 14 female), diarrhoea-variant (31.9%; 16 male and 15 female) or alternated alveus-variant (47.5%; 19 male and 27 female) IBS. The severity of the alveus disturbances was scored according to a 5-point semi-quantitative scale (0 = none; 1 = minimum; 2 = mild; 3 = moderate; 4 = severe).

The IBS patients were then asked to undergo a BTLact to check the presence of SIBO; only 97 patients accepted, while the remaining 18 patients refused further investigations. At the evening before the BTLact, the patients were required to eat only boiled rise with no sausage or cheese, and grilled meat, and to drink only no-gas water. If constipation was present, the dietary prescriptions were extended to the 3 d preceding the exam. On the day of test, the patients were completely fasted, and smoking was forbidden to all patients (smokers included). Immediately before the test, a sample of expired air was taken to assay the basal H_2 concentrations in the still fasted subjects; then 25 g of lactulose was administered and the expired air was sampled every 30 min in the next three consecutive hours.

A positive test required an early increase of H_2 concentration in the expired air higher than 20 ppm over basal values within 90 min of the oral administration of lactulose, followed by a second distinct peak after additional 15 min or more[9].

The patients, who had a positive BTLact, received a diagnosis of SIBO and were treated with Rifaximin (Normix®, Alfa Wassermann) at the daily dose of 1200 mg for 7 d. Three weeks after the end of the treatment, the BTLact was repeated and the alveus disturbances were scored again.

Statistical analysis

The demographic characteristics of the patients were described as means and standard deviations (min-max ranges), or frequencies when appropriate. The frequencies of symptoms observed in patients with diagnosis of SIBO and IBS were compared using χ^2 test; the frequency of positive BTLact was analysed by the Fisher's exact test.

RESULTS

The 97 patients with diagnosis of IBS, who accepted BTLact, were 41 male and 56 female. The BTLact was found positive in 54 (55.6%) patients; in particular, the test was positive in 61.3% of patients with diarrhoea-, 52.2% of patients with constipation- and 52% of patients with alternated constipation/diarrhoea-variant IBS. There was no significant difference in the frequencies of IBS variants between patients with positive or negative BTLact. The global symptomatological score was not different between the patients with positive or

Table 1 Demographic characteristics of patients with a new diagnosis of IBS (*n* = 97)

Demographic characteristics	*n*	Breath test at screening	
		Positive (*n* = 54)	Negative (*n* = 43)
Sex (M/F, mean ± SD)	40/57 (40.7 ± 16.2)		
IBS variants *n* (%)			
Chronic diarrhoea	31 (31.9)	19 (35.2)	12 (27.9)
Stipsis	20 (20.6)	11 (20.4)	9 (20.9)
Alternated stipsis/diarrhoea	46 (47.5)	24 (44.4)	22 (51.2)
Symptoms score			
Chronic diarrhoea	2.2 ± 0.8	2.2 ± 0.7	2.1 ± 0.6
Stipsis	2.3 ± 0.9	2.4 ± 0.8	2.1 ± 0.9
Alternated stipsis/diarrhoea	2.0 ± 1.2	2.3 ± 0.9	1.7 ± 0.6[a]

[a]Statistically significant difference between groups, $P = 0.019$.

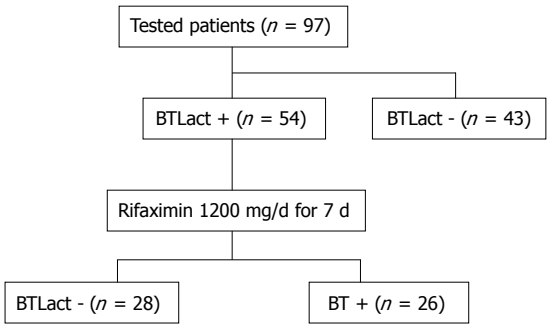

Figure 1 Flow-chart of the activities performed, and the results achieved during the observational study. BTLact: Breath test with lactu-lose.

negative BTLact, except for patients with the alternated constipation/diarrhoea-variant IBS; among these patients, the symptomatological score was significantly higher in the BTLact-positive subjects compared to BTLact-negative subjects (Table 1).

The 54 BTLact-positive patients were treated with Rifaximin, and the BTLact was repeated 3 wk after end of the treatment. In 28 patients, the BTLact turned to be negative, thus showing that the antibiotic was able to significantly control the bacterial overgrowth in the small intestine; in these patients a statistically significant reduction of the symptomatological score from 2.3 ± 0.6 to 0.9 ± 0.8 was also achieved ($P = 0.003$). On the contrary, the remaining 26 patients still had a positive BTLact, and no change in the symptomatological score was observed (2.3 ± 0.6 *vs* 2.3 ± 0.6). No treatment-related adverse effect was observed. A flow-chart of the study is shown in Figure 1.

DISCUSSION

Bacterial flora consisting of Gram-positive and Gram-negative germs, aerobes and anaerobes, is distributed along the GI tract in varying quantities from zero to a maximum of 10^{12}/mL of endoluminal aspirate. This bacterial ecosystem counterbalances with the ecological niche of the host organism and harmonizes with the various digestive, secretory, motor, absorption and sensitivity functions of the entire intestine.

This dynamic equilibrium between environment, bacterial flora and host may be interrupted due to a variety of complex reasons, leading to quantitative and qualitative modifications of the normal intestinal microbial flora that can cause SIBO[10]. SIBO thus represents an invasion of the small intestine, from the upper part by pathogenic strains of oro-alimentary origin, and from the lower part by colo-fecal germs through an incontinent Bauhin's valve.

The SIBO has various clinical and biological presentations: chronic diarrhea, malabsorption syndrome and exudative enteropathy are the main criteria of diagnosis[11]; the syndrome is characterized by an increase of overall bacterial burden in biotope > 10^5 CFU/mL in adults and > 10^4 CFU/mL in children, emergence of different species of enterobacteria, bacteroides, clostridia and fusobacteria in small intestine. Microecological changes are accompanied by B_{12} vitamin deficiency anemia, hypovitaminosis, protein deficiency, translocation of bacteria and their toxins from intestine in blood, emergence of endotoxinemia and possible generalization of infection[12].

Our survey suggested that, based on the results of a BTLact, SIBO was present in about 56% of newly diagnosed IBS patients in our geographical area. Our study has significant limitations, since it was open label; moreover, some clinicians do not consider the BTLact as a gold standard for diagnosis of SIBO and they recommend indirect parameters, like serum vitamine B_{12} levels and folate levels, as main indicator of SIBO. Notwithstanding, our observations find a confirmation in the prevalence of abnormal BTLact recently reported by other authors[13].

The treatment of SIBO must be firstly focused on the correction of wrong food and dietary habits that usually underlying the disorder (e.g. excessive use of fast-food), and then to the reduction of bacterial colonization of small intestine by means of antibiotics[14-16]. In this regard, the use of locally acting, non-absorbable antibiotics would be particularly useful in reducing immediately the bacterial count waiting for the slow-acting beneficial effects of dietary measures. Decontamination of the small intestine is more successful when probiotics are prescribed (both after antibiotics and independently), which suppress the opportunistic flora, protect the mucous coat, improve digestion and arrest diarrhea[17].

Our study demonstrated that a 7-d treatment with Rifaximin determined the negativization of BTLact in about 50% of treated patients. Although the treatment was very short (7 d) and no long-term follow-up available, our data seem to confirm other experiences reported in the most recent literature by Majewski *et al*[3] that a daily dose of 800 mg Rifaximin for 4 wk significantly reduced the symptoms in 20 patients with IBS and led to a negative BT in almost half of patients. Moreover, in another series of 23 patients with SIBO and positive BT, administration of Rifaximin 1200 mg/d for 7 d followed by treatment with probiotics led to a negative BT in 19 (82.6%) cases and significantly reduced the peak in hydrogen concentrations in the expired air from 40.9 ± 20.4 to 4.78 ± 8.42 ppm[2]; Rifaximin was also more

effective than chlortetracycline in improving symptoms in patients with SIBO and IBS[18]. More evidences on the efficacy of Rifaximin have been reported in patients with SIBO and acute diverticulitis of colon[19], and patients with SIBO and celiac disease[20].

In this study, 48% of patients treated with Rifaximin failed to achieve a clinical benefit and turn the BTLact to negative. On the other hand, the difficulty in identifying the specific bacterial population and the affected part of the digestive tract by SIBO prevents the possibility of using targeted antibiotics; the recommendation is to use a broad-spectrum antibiotic therapy, capable of eradicating aerobes and anaerobes, preferably with a topical rather than a general action. Valuable alternatives to Rifaximin that have been proven to be effective in the treatment of SIBO are norfloxacin and amoxicillin-clavulanic acid[21], levofloxacin and/or metronidazole[22], gentamycin[23], trimethoprim/sulfamerazine and polymyxin[24], and chlortetracycline[18].

COMMENTS

Background

The symptoms of irritable bowel syndrome (alternated stipsis and diarrhoea) may be frequently mimicked by an overgrowth of the bacteria that normally reside in the intestine.

Research frontiers

The authors aimed to establish which is the actual incidence of such a bacterial overgrowth among patients with symptoms of IBS, and to get more information on how to treat this disturbance.

Innovations and breakthroughs

The investigation has shown that more than 50% of patients with an earlier diagnosis of IBS, suffering indeed of an intestinal bacterial overgrowth, can be treated by means of an appropriate treatment.

Applications

The intestinal bacterial overgrowth can be detected by means of a specific test ("hydrogen breath test"), which is encouraged to be performed by gastroenterologists.

Peer review

The paper provides an interesting information on the frequency of the intestinal bacterial contamination and overgrowth, which is due to the modern alimentary habits and produces symptoms that may be confused with other gastrointestinal diseases; a simple test, such as the measurement of hydrogen in the expired air, can identify the disorder and allow for the right pharmacological treatment.

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BRIEF ARTICLES

Prognosis of hepatocellular carcinoma accompanied by microscopic portal vein invasion

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Abstract

AIM: To investigate the prognostic factors in patients with hepatocellular carcinoma (HCC) accompanied by microscopic portal vein invasion (PVI).

METHODS: Of the 267 patients with HCC undergoing hepatic resection at Aso Iizuka Hospital, 71 had PVI. After excluding 16 patients with HCC that invaded the main trunk and the first and second branches of the portal vein, 55 patients with microscopic PVI were enrolled.

RESULTS: The patients with HCC accompanied by microscopic invasion were divided into two groups: solitary PVI (PVI-S: n = 44), and multiple PVIs (PVI-M: n = 11). The number of portal vein branches invaded by tumor thrombi was 5.4 ± 3.8 (2-16) in patients with PVI-M. In cumulative survival, PVI-M was found to be a significantly poor prognostic factor (P = 0.0019); while PVI-M and non-anatomical resection were significantly poor prognostic factors in disease-free survival (P = 0.0213, and 0.0115, respectively). In patients with PVI-M, multiple intrahepatic recurrence was more common than in the patients with PVI-S (P = 0.0049). In patients with PVI-S, non-anatomical resection was a significantly poor prognostic factor in disease-free survival (P = 0.0370). Operative procedure was not a significant prognostic factor in patients with PVI-M.

CONCLUSION: The presence of PVI-M was a poor prognostic factor in patients with HCC, accompanied by microscopic PVI. Anatomical resection is recommended in these patients with HCC. Patients with HCC and PVI-M may also be good candidates for adjuvant chemotherapy.

Key words: Hepatocellular carcinoma; Microscopic portal vein invasion; Hepatectomy; Prognosis; Recurrence

Peer reviewer: Roberto Testa, Professor, Department of Internal Medicine, University of Genoa, Viale Benedetto XV 6, Genoa 16132, Italy

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INTRODUCTION

Hepatocellular carcinoma (HCC) is a malignant tumor with periportal venous metastasis. Vascular invasion, especially portal vein invasion (PVI), is a major determinant of outcome after hepatic resection in patients with HCC[1-7].

Magnetic resonance imaging (MRI) and ultrasonography can detect tumor invasion of the major branches of the portal or hepatic veins, 81%-95% of the time[8-10]. However, the presence of microscopic PVI, limited to the subsegmental portal vein branches, cannot be diagnosed before hepatic resection. Recently reported predictors of microscopic PVI include size, number, and histological grade of tumors, as well as serum

level of des-γ-carboxy prothrombin (DCP)[11-13]. Miyata *et al*[14] have demonstrated that tumorous arterioportal (A-P) shunt formation on computed tomography (CT) during hepatic arteriography is an important predictive value for PVI. A recent study by Nishie *et al*[15] has shown that an area exhibiting low attenuation on CT during arterio-portography, and high attenuation on CT during hepatic arteriography around the tumor, is a good predictor of PVI. Thus, recent studies have suggested that the presence of microscopic PVI can be predicted.

Nevertheless, the prognostic factors of HCC with microscopic PVI have remained elusive and the operative procedures for this type of HCC have not been determined. In the present study, we evaluated the prognostic factors in cumulative and disease-free survival in patients with HCC, accompanied by microscopic PVI.

MATERIALS AND METHODS

Patients

From April 1992 to December 2005, 267 patients underwent their first liver resection for HCC at the Department of Hepatogastro-enterological Surgery at Aso Iizuka Hospital in Japan. From a retrospective database, 55 patients were enrolled in this study, according to the following criteria: (1) an absence of HCC invading the main trunk and the first and second branches of the portal vein, upon preoperative radiological evidence and intraoperative findings; (2) no remnant cancer after surgery, as confirmed by ultrasonography, CT and/or MRI; and (3) the presence of microscopic PVI upon histological examination.

There were 41 male and 14 female patients with an average age of 64 years (median: 66 years). Among these patients, 35 (64 %) were infected with hepatitis C virus, which leads to chronic liver disease. The indocyanine green retention test at 15 min was 15.5% ± 10.2%. On pathological examination, the tumor size was 5.0 ± 1.1 cm and the main grade of cancer cell was moderately differentiated in 38 patients (69%) and poorly differentiated in 17 (31%). Microscopic intrahepatic metastasis was found in 24 patients (44%).

Methods

The prognostic factors were examined in cumulative and disease-free survival, using the following variables: age (older or younger than 67 years); gender (male *versus* female); platelet numbers (greater than *versus* less than or equal to 150 000/mm^3); serum albumin levels (greater than *versus* less than or equal to 3.8 g/dL); tumor size (greater than *versus* less than or equal to 4.2 cm); serum levels of alpha-fetoprotein (AFP) (greater than *versus* less than or equal to 28 ng/mL); DCP (greater than *versus* less than or equal to 300 mAU/mL); operative procedures (anatomical *versus* non-anatomical resection); histological grading of cancer cell differentiation (moderate *versus* poor); presence of intrahepatic metastases (negative *versus* positive); and microscopic PVI (solitary or multiple). The measurement of serum DCP has been described previously[13]. The measurement of serum DCP was started at our hospital in 1999 and was therefore only available in the latest 36 patients.

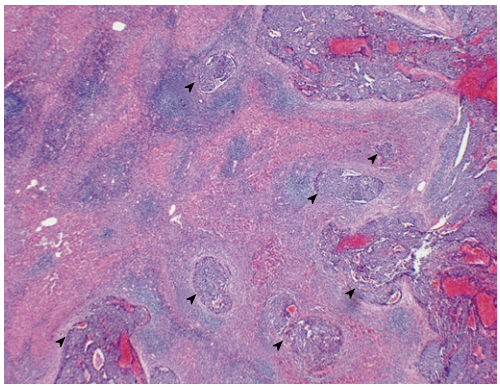

Figure 1 Multiple microscopic PVI surrounding a tumor (arrow heads). HE staining (Original magnification, × 20).

Anatomical resection included hemi-hepatectomy, segmentectomy, and subsegmentectomy, based on Couinaud's classification. Non-anatomical resection was partial hepatectomy, including the tumor.

Histological study

All of the resected specimens were cut into serial 5-10-mm thick slices and fixed in 10% formalin. After macroscopic examination, the slice with the greatest dimension was trimmed for paraffin blocks and cut into 4-μm microscopic sections. The slices were then stained with hematoxylin and eosin (HE). When clusters of cancer cells were present in the extra tumoral portal vein, accompanied with bile duct and hepatic artery, it was defined as positive for extra tumoral PVI. When more than two clusters of cancer cells were present in different portal vein branches, it was defined as multiple PVI (PVI-M) (Figure 1). When only one cluster was present in a single portal vein branch, it was defined as solitary PVI (PVI-S).

Follow-up strategy and recurrence pattern

After discharge, all patients were examined for recurrence by ultrasonography and tumor markers, such as AFP and DCP every month, and by CT every 6 mo. When recurrence was suspected, additional examinations such as hepatic angiography were performed. The recurrence pattern was determined by ultrasonography, CT, MRI and hepatic angiography and was defined as previously reported[7]. Briefly, none was the absence of HCC recurrence, nodular recurrence was fewer than four recurrent nodules, and multiple recurrence was four or more recurrent nodules.

Impact of operative procedures in patients with PVI-S and PVI-M

In patients with PVI-S and PVI-M, the impact of operative procedures (non-anatomical *versus* anatomical resection) was compared on cumulative and disease-free survival.

Table 1 Univariate analysis of clinicopathological prognostic factors for cumulative survival rate

Factors	Survival rate (%)			*P* value
	1 yr	3 yr	5 yr	
Age (yr)				
< 67 (*n* = 27)	80.6	60.4	42.1	0.9546
⩾ 67 (*n* = 28)	96.6	75.7	50.0	
Gender				
Male (*n* = 41)	92.6	72.3	45.2	0.7614
Female (*n* = 14)	78.6	50.0	42.9	
Platelets (10^4/mm^3)				
< 1.5 (*n* = 27)	92.6	61.7	46.6	0.7341
⩾ 1.5 (*n* = 28)	85.7	67.5	46.0	
Albumin (g/dL)				
< 3.9 (*n* = 27)	100	76.9	47.2	0.6123
⩾ 3.9 (*n* = 28)	79.0	56.8	47.5	
Tumor size (cm)				
< 4.3 (*n* = 27)	92.4	72.8	35.9	0.8752
⩾ 4.3 (*n* = 28)	85.7	60.7	52.0	
AFP (ng/mL)				
0-27 (*n* = 28)	96.4	71.3	55.4	0.1001
> 27 (*n* = 27)	81.2	61.6	35.8	
DCP (mAU/mL)				
0-300 (*n* = 18)	94.1	75.1	75.1	0.1834
⩾ 300 (*n* = 18)	83.3	55.6	55.6	
Operative procedure				
Anatomical resection (*n* = 32)	90.5	74.2	51.4	0.0620
Non-anatomic resection (*n* = 23)	87.0	55.9	39.5	
Tumor grade of differentiation				
Moderate (*n* = 38)	97.3	70.0	54.9	0.1076
Poor (*n* = 17)	70.6	58.8	30.3	
IM (-) (*n* = 31)	96.8	66.7	56.5	0.2625
IM (+) (*n* = 24)	79.2	66.7	32.8	
PVI-S (*n* = 44)	97.7	76.5	51.2	0.0019
PVI-M (*n* = 11)	54.5	27.3	27.3	

IM: Intrahepatic metastasis.

Statistical analysis

All data were expressed as mean ± SD. The χ^2 test of independence was used with categorical variables. The continuous variables were divided by their median values. The survival and disease-free survival curves were generalized using the Kaplan-Meier method and then compared using the log-rank test. The Stat view software (Version 4.11; Abacus Concepts Inc., Berkeley, CA, USA) was used for the analysis on a Macintosh computer. $P < 0.05$ was considered to be statistically significant.

RESULTS

Histological examination of PVI-S and PVI-M

Of the 267 patients, 55 (21%) had microscopic PVI. The overall incidence of PVI-S was 16% (44 patients) and that of PVI-M was 4% (11 patients). Of the 55 patients with PVI, the 11 patients with PVI-M represented 20%. The number of portal vein branches invaded by tumor thrombi was 5.4 ± 3.8 (2-16) on the slices, stained with HE in patients with PVI-M.

Significant prognostic factors in cumulative survival

The overall survival after hepatectomy in 55 patients with microscopic PVI was 89.0% at 1 year, 66.6% at 3 years, 46.0% at 5 years, and 36.1% at 10 years (Figure 2).

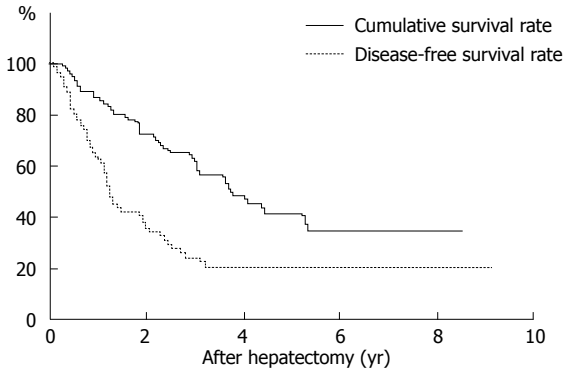

Figure 2 Cumulative and disease-free survival rate in patients with HCC, accompanied by microscopic PVI. The overall cumulative and disease-free survival after hepatectomy in 55 patients was 89.0% and 68.7% at 1 year, 66.6% and 27.3% at 3 years, 46.0% and 22.7% at 5 years.

The median survival was 4.6 ± 0.6 years. Among 11 clinicopathological factors, only the extent of PVI was significant (Table 1). Thus, the cumulative survival curve for patients with PVI-M was significantly worse than that of those with PVI-S ($P = 0.0019$). The median survival of patients with PVI-S and PVI-M was 5.4 ± 1.8 and 1.3 ± 0.7 years, respectively. In operative procedures, the survival rate for anatomical resection tended to be better than that for non-anatomical resection, although the difference was not significant ($P = 0.0620$).

Significant prognostic factors in disease-free survival

The disease-free survival in 55 patients was 68.7% at 1 year, 27.3 % at 3 years, 22.7 % at 5 years, and 22.7 % at 10 years (Figure 2). Among 11 clinicopathological factors, the extent of PVI and operative procedures were significant (Table 2). The disease-free survival curve for patients with PVI-M was significantly worse than that for patients with PVI-S ($P = 0.0072$). The median disease-free survival in PVI-S and PVI-M was 1.5 ± 0.8 and 0.4 ± 0.2 years, respectively. In operative procedures, the disease-free survival rate for anatomical resection was significantly better than that for non-anatomical resection ($P = 0.0074$). The median disease-free survival for anatomical and non-anatomical resection was 2.0 ± 0.2 and 0.8 ± 0.1 years, respectively.

Comparison of PVI-S and PVI-M recurrence patterns

A comparison of the recurrence pattern is shown in Table 3. The incidence of multiple recurrence in patients with PVI-M was 82% and 30% in patients with PVI-S ($P = 0.0049$).

Impact of operative procedures in patients with PVI-S and PVI-M

In patients with PVI-S, non-anatomical resection tended to be a poor prognostic factor for cumulative survival ($P = 0.0782$), while non-anatomical resection was significantly poor prognostic factor in disease-free survival ($P = 0.0370$) (Table 4). In patients with PVI-M, operative procedures were not significant in disease-free survival (Table 5).

Table 2 Univariate analysis of clinicopathological prognostic factors for disease-free survival rate

Factors	Survival rates (%)			*P* value
	1 yr	3 yr	5 yr	
Age (yr)				
< 67 (*n* = 27)	54.7	31.9	31.9	0.7438
⩾ 67 (*n* = 28)	72.5	21.3		
Gender				
Male (*n* = 41)	68.7	27.3	22.7	0.2716
Female (*n* = 14)	48.6	19.4	19.4	
Platelets (10^4/mm^3)				
< 1.5 (*n* = 27)	52.8	7.9	7.9	0.1703
⩾ 1.5 (*n* = 28)	74.2	33.7	33.7	
Albumin (g/dL)				
< 3.9 (*n* = 27)	61.5	22.6	16.9	0.8555
⩾ 3.9 (*n* = 28)	65.8	29.4	29.4	
Tumor size (cm)				
< 4.3 (*n* = 27)	62.9	35.0	35.0	0.4474
⩾ 4.3 (*n* = 28)	64.8	17.0	11.3	
AFP (ng/mL)				
0-27 (*n* = 28)	59.1	39.4	39.4	0.1692
> 27 (*n* = 27)	68.0	14.6	9.7	
DCP (mAU/mL)				
< 300 (*n* = 18)	72.5	43.5	43.5	0.2995
⩾ 300 (*n* = 18)	63.0	21.0	14.0	
Operative procedure				
Anatomical resection (*n* = 32)	82.3	33.0	33.0	0.0074
Non-anatomical resection (*n* = 23)	40.9	17.0	11.4	
Tumor grade of differentiation				
Moderate (*n* = 38)	69.3	36.7	31.4	0.0661
Poor (*n* = 17)	52.9	7.4	7.4	
IM (-) (*n* = 31)	67.1	28.2	23.5	0.4761
IM (+) (*n* = 24)	59.8	22.1	22.1	
PVI-S (*n* = 44)	74.6	27.4	23.5	0.0072
PVI-M (*n* = 11)	20.0	20.0		

DISCUSSION

The incidence of microscopic PVI has been reported to be more than 20% in resected HCC[1,2]. Even in small HCCs, up to 2 cm in diameter, the incidence of PVI is 15%[3]. In this study, the incidence of PVI was found in 55 of 267 (20%) patients.

Vascular invasion, especially PVI, is a major determinant of the outcome after hepatic resection in patients with HCC[1-7]. In the present study, the survival rate was poorer for HCC patients with PVI than those without (data not shown). Nevertheless, the prognosis of patients with PVI varied. With regard to recurrence patterns, 18 (33%) of the 55 patients with HCC accompanied by PVI had no recurrence, and 22 patients (40%) had multiple recurrence. Clearly, the outcome in patients with no recurrence was better than that of patients with multiple recurrence.

Determination of the prognostic factors in patients with PVI is important for postoperative therapeutic strategy. There has been no study of prognostic factors in patients with HCC accompanied by PVI. In the present study, detailed histological examination of resected specimens revealed that PVI-M was a significantly poor prognostic factor after hepatectomy for cumulative and disease-free survival. With regard to operative procedures, anatomical resection tended to improve survival rates and significantly improve disease-free rates.

Table 3 Comparison of recurrence patterns for 55 HCC patients with single or multiple PVI *n* (%)

Recurrence pattern	None	Nodular	Multiple
PVI-S (*n* = 44)	16 (36)	15 (34)	13 (30)
PVI-M (*n* = 11)	2 (18)	0	9 (82)

The incidence of multiple recurrence in PVI-M was significantly higher than that in PVI-S ($P = 0.0049$).

Table 4 Impact of operative procedures (non-anatomical *versus* anatomical resection) was examined in patients with PVI-S, depending on cumulative survival rate and disease-free survival rates

Factors	Survival rates (%)			*P* value
	1 yr	3 yr	5 yr	
Cumulative survival				
Anatomical resection (*n* = 26)	96.2	83.9	55.0	0.0782
Non-anatomical resection (*n* = 18)	100	65.8	46.1	
Disease-free survival				
Anatomical resection (*n* = 26)	90.9	32.2	32.2	0.0370
Non-anatomical resection (*n* = 18)	52.9	22.1	14.7	

Table 5 Impact of operative procedures (non-anatomical *versus* anatomical resection) was examined in patients with PVI-M, depending on cumulative survival and disease-free survival rates

Factors	Survival rates (%)			*P* value
	1 yr	3 yr	5 yr	
Cumulative survival				
Anatomical resection (*n* = 6)	66.7	33.3	33.3	0.4497
Non-anatomical resection (*n* = 5)	40.0	20.0	20.0	
Disease-free survival				
Anatomical resection (*n* = 6)	40.0	20.0	20.0	0.2651
Non-anatomical resection (*n* = 5)	0			

Histologically, the number of portal vein branches invaded by tumor thrombi was 5.4 ± 3.7 (2-16) in patients with PVI-M. Although PVI was limited to the subsegment of the liver, multiple portal vein branches that surrounded the tumor were invaded. In these patients, the biological behavior of HCC with PVI-M may be similar to that of HCC, with invasion to the first branches or main trunks of the portal vein. The survival rate in patients with PVI-M was only 54.5% at 1 year after hepatectomy. The mean survival and disease-free survival after hepatectomy in patients with PVI-M was 1.3 and 0.4 years, respectively. Multiple recurrence was more common in patients with PVI-M than those with PVI-S. This clinical outcome was similar to that previously shown for patients with HCC, accompanied by portal vein thrombi of the first branches or main trunks[16,17].

Anatomical resection of the liver significantly improved the disease-free rates for HCC with PVI in the present study. Hasegawa *et al*[18] have reported that anatomical resection, such as segmentectomy and subsegmentectomy for HCC, is a reasonable treatment option and yields more favorable results than non-anatomical resection. A recent comparison of the

outcomes of anatomical subsegmentectomy and non-anatomical minor hepatectomy for single HCC, based on a Japanese nationwide survey, recommends anatomical resection, especially when the size of HCC ranges 2-5 cm[19]. In our study, non-anatomical resection was a significantly poor prognostic factor in disease-free survival and tended to be a poor prognostic factor in cumulative survival in patients with PVI-S. Therefore, anatomical resection is preferable in patients with HCC accompanied by PVI. Recent studies have demonstrated that HCC with PVI can be predicted by several factors[11-15]. Therefore, patients with HCC that have high risk factors for PVI preoperatively should be recommended for anatomical resection. To clarify this hypothesis, further examination is necessary.

In PVI-M patients, there was no significant difference in the outcome between anatomical and non-anatomical resection. Recently, in patients with portal vein thrombi in major portal branches, adjuvant chemotherapy has been reported to be effective following hepatectomy and thrombectomy[16,17,20,21]. Patients with HCC and PVI-M may also be good candidates for adjuvant chemotherapy.

In conclusion, in patients with HCC, accompanied by microscopic PVI, the presence of PVI-M is a poor prognostic factor. In PVI-S patients, anatomical resection is preferable to non-anatomical resection. Patients with HCC and PVI-M may be good candidates for adjuvant chemotherapy. Further studies aimed at improving the outcome of patients with PVI after hepatectomy are necessary.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is a malignant tumor with periportal venous metastasis. Vascular invasion, especially portal vein invasion (PVI), is a major determinant of outcome after hepatic resection in patients with HCC. Nevertheless, the prognostic factors of HCC with microscopic PVI have remained elusive and the operative procedures for this type of HCC have not been determined.

Research frontiers

The prognostic factors in cumulative and disease-free survival in patients with HCC, accompanied by microscopic PVI, were evaluated in the present study.

Innovations and breakthroughs

The presence of multiple PVI (PVI-M) was a poor prognostic factor in patients with HCC, accompanied by microscopic PVI. Anatomical resection is recommended in these patients with HCC. Patients with HCC with PVI-M may be good candidates for adjuvant chemotherapy.

Applications

In patients with HCC, accompanied by microscopic PVI, the presence of PVI–M is a poor prognostic factor. In solitary PVI patients, anatomical resection is preferable to non-anatomical resection. Patients with HCC and PVI-M may be good candidates for adjuvant chemotherapy.

Terminology

When clusters of cancer cells were present in the extra tumoral portal vein, accompanied with bile duct and hepatic artery, it was defined as positive for extra tumoral PVI. When more than two clusters of cancer cells (PVI) were present in different portal vein branches, it was defined as PVI-M. When only one cluster was present in a single portal vein branch, it was defined as solitary PVI.

Peer review

This paper was correctly planned to investigate the prognostic factors in patients with HCC accompanied by microscopic PVI. This research is of clinical importance in post-resection management of patients with HCC. The results provide robust clinical evidence to suggest firm scientific conclusions.

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BRIEF ARTICLES

Adjuvant percutaneous radiofrequency ablation of feeding artery of hepatocellular carcinoma before treatment

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Abstract

AIM: To evaluate the feasibility and efficacy of percutaneous radiofrequency ablation (RFA) of the feeding artery of hepatocellular carcinoma (HCC) in reducing the blood-flow-induced heat-sink effect of RFA.

METHODS: A total of 154 HCC patients with 177 pathologically confirmed hypervascular lesions participated in the study and were randomly assigned into two groups. Seventy-one patients with 75 HCCs (average tumor size, 4.3 ± 1.1 cm) were included in group A, in which the feeding artery of HCC was identified by color Doppler flow imaging, and were ablated with multiple small overlapping RFA foci [percutaneous ablation of feeding artery (PAA)] before routine RFA treatment of the tumor. Eighty-three patients with 102 HCC (average tumor size, 4.1 ± 1.0 cm) were included in group B, in which the tumors were treated routinely with RFA. Contrast-enhanced computed tomography was used as post-RFA imaging, when patients were followed-up for 1, 3 and 6 mo.

RESULTS: In group A, feeding arteries were blocked in 66 (88%) HCC lesions, and the size of arteries decreased in nine (12%). The average number of punctures per HCC was 2.76 ± 1.12 in group A, and 3.36 ± 1.60 in group B (P = 0.01). The tumor necrosis rate at 1 mo post-RFA was 90.67% (68/75 lesions) in group A and 90.20% (92/102 lesions) in group B. HCC recurrence rate at 6 mo post-RFA was 17.33% (13/75) in group A and 31.37% (32/102) in group B (P = 0.04).

CONCLUSION: PAA blocked effectively the feeding artery of HCC. Combination of PAA and RFA significantly decreased post-RFA recurrence and provided an alternative treatment for hypervascular HCC.

Key words: Hepatocellular carcinoma; Feeding artery; Radiofrequency ablation; Recurrence; Color Doppler flow imaging

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer in the world (564000 cases per year) and the third most frequent cause of cancer-related death[1]. Surgical resection is considered to be potentially curative therapy. However, only about 20% of HCC patients are eligible for resection[1,2]; the remainder are ineligible because of multifocal tumors, advanced tumors, tumor location precluding complete resection, or poor hepatic functional reserve. Therefore, a variety of imaging-guided tumor ablation therapies such as ethanol injection, microwave coagulation, percutaneous radiofrequency ablation (RFA) and laser ablation are often considered as alternative options[3-6]. Among them, RFA has been used increasingly as a safe technique for treating hepatic tumors[7-9]. However, for hypervascular HCC, RFA appears less effective because of a blood-flow-induced heat sink effect, which might

cause incomplete ablation or recurrence[10]. Transcatheter arterial chemoembolization (TACE) can reduce blood supply of HCC by occlusion of tumor arteries, and the efficacy of combination TACE and RFA has been confirmed[11,12]. Treatment difficulty remains for those patients who cannot tolerate or are ineligible for TACE because of liver cirrhosis or difficulty in manipulation of vessels with abnormal curvature that have resulted from surgical resection and liver transplantation.

We hypothesized that, if percutaneous ablation of the feeding artery (PAA) of HCC could block or reduce the blood flow of HCC, the ablation volume of coagulation necrosis of subsequent RFA of the tumor would be increased. To the best of our knowledge, the application of PAA in the treatment of hypervascular HCC has not been reported in a large number of patients. In the present study, we evaluated the feasibility and adjuvant value of PAA performed before routine RFA treatment (PAA-RFA) of hypervascular HCC.

MATERIALS AND METHODS

Patients

From January 2003 to June 2007, patients with HCC who met the entry criteria and agreed to participate were included in the study. The inclusion criteria for the patients were: pathologically confirmed HCC lesions, ineligibility for surgical resection or TACE, tumor size > 3 cm, no significant tumor direct invasion of adjacent organs, tumor not invading the main bile duct or being obviously exophytic, tumor's feeding artery visible on color Doppler flow imaging (CDFI), prothrombin time ratio > 50%, or platelet count > 60×10^9/L. Exclusion criteria were: tumor thrombus in main or lobar portal vein system, extrahepatic metastasis, or Child-Pugh class C liver cirrhosis.

Each patient who participated in the study was assigned a random number of 1 or 2. The patients with number 1 were allocated to group A, which was treated with PAA-RFA, and those with number 2 were allocated to group B, which was treated with routine RFA[13].

This study was performed with the approval of the ethics committee and informed consent was obtained from each patient after the nature of the procedure had been fully explained.

Equipment

The RFA system used in this study was a 460-kHz generator, 150 W output power (Model 1500; RITA Medical Systems, Mountain View, CA, USA). The expandable electrodes consisted of an outer 14-gauge, 15-cm long outer insulated needle, and nine prongs that were deployed and retracted by a movable hub, with deployment diameter ranging from 3 to 5cm. Twenty minutes were required to produce a 5-cm ablation sphere during RFA. Track ablation was performed when withdrawing the RFA electrode in all patients, to avoid implantation metastasis and hemorrhage.

Real-time ultrasound (US) systems (Aloka 5500 and Aloka α-10, Tokyo, Japan) were used for scanning with 3.5-5.0-MHz convex probes with needle guide devices. Computed tomography (CT) was performed with a Plus 4 scanner (Siemens, Germany) with 5 mm collimation and a table speed of 7.5 mm/s. A total of 100 mL of non-ionic contrast material (300 mg iodine/mL, Omnipaque; Amersham, Shanghai, China) was administered at a rate of 3 mL/s with a power injector (OP 100; Medrad, Pittsburgh, PA, USA). Images were acquired before contrast material injection and 25 and 60 s after the administration of intravenous contrast material, during the hepatic arterial and portal venous phases, respectively.

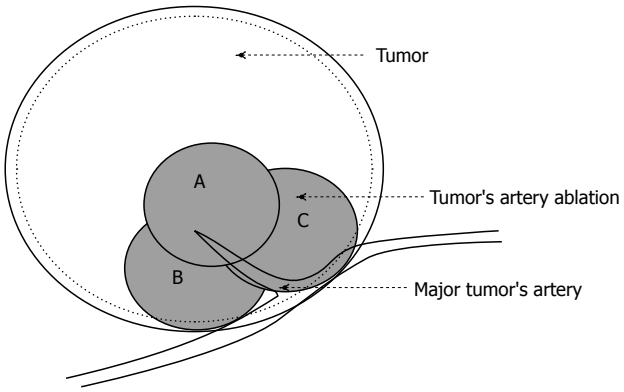

Figure 1 Schematic drawing of PAA using three small ablation foci at the area where the feeding artery entered the tumor, to block the blood supply of HCC.

PAA and RFA

All RFA was performed by two radiologists (M.H.C. and K.Y.) who had more than 10 years' experience of US-guided interventional procedures. Before RFA, the patients were examined by ultrasound and contrast CT or magnetic resonance imaging, and the size, shape and border of the tumor were determined, mainly based on US scans.

CDFI was used to identify the major feeding artery and guide the RFA needle to puncture the area where the artery entered the tumor. This area was ablated with 2-3 overlapping, high-energy ablation foci (2-3 cm each in diameter) in different direction or depths (Figure 1). The flow rate and vessel size of the feeding artery was measured before and immediately after PAA to evaluate the blood supply. After PAA, routine RFA treatment of the tumor was performed immediately, and the ablated area covered 0.5-1 cm beyond the tumor margin. As a result of the hypervascular character of the lesions, special attention was paid to using a slow withdrawal process of the electrode needle to avoid bleeding.

Patients in group B underwent routine RFA treatment using multiple overlapping ablation spheres to cover the tumor and the safe margin beyond the tumor of 0.5-1 cm[13]. During RFA, moderate intravenous sedation was induced with 2.5-5.0 mg midazolam (Roche, Basel, Switzerland) and 50-100 μg fentanyl (Fentaini; Renfu, Yichang, China). Local infiltration anesthesia was induced by 5-15 mL 1% lidocaine (Liduokayin; Yimin, Beijing, China). If patients with tumors adjacent to the diaphragm and hepatic hilum experienced local and

right shoulder pain when the ablation was extended, intravenous infusion of propofol (Diprivan, 1-2 mg/kg; Zeneca, Macclesfield, UK) was given for temporary anesthesia enhancement.

The patients were conscious when the RFA electrode was placed, and vital signs and oxygen saturation were monitored continuously during the procedure. After RFA, the patients underwent close medical observation and were rescanned within 1-2 h after the procedure to detect any bleeding in the liver or the peritoneal cavity. All patients stayed in the hospital overnight.

Any adverse events were evaluated and recorded. Major complications were defined as those that, if left untreated, might have threatened the patient's life, led to substantial morbidity and disability, or resulted in hospital admission or a substantially lengthened hospital stay. All other complications were considered minor.

Assessment of therapeutic efficacy

Treatment response was assessed by contrast-enhanced spiral CT at 1 mo after RFA and complete response was considered to be achieved if the CT scans revealed: (1) the ablation zone was beyond the original tumor borders; (2) the margin of the ablation zone was clear and smooth; and (3) no arterial enhancement or abnormal wash-out was detected within or around the tumor. Subsequently, the patients were followed-up with serum alpha-fetoprotein (AFP) measurement, abdominal US, and contrast-enhanced CT every 2-3 mo in the first year, and then every 4-6 mo thereafter. All patients were followed-up for at least 6 mo. Recurrence was defined as enhancement within or at the periphery of the ablated area in the follow-up CT scan. Recurrent HCC was treated with another session of RFA. All CT scans were reviewed by two radiologists with more > 10 years experience, who were unaware of patient clinical data or treatment assignment.

Statistical analysis

Differences in complete necrosis rate at 1 mo and recurrence rate at 6 mo were analyzed by χ^2 and *t* tests where appropriate. The recurrence rate was determined by log-rank tests, and multivariate hazard ratio was calculated using the Cox proportional hazard model. The Kaplan-Meier estimate of the cumulative recurrence rates over time was also carried out. All *P* values were two-sided, and $P \leqslant 0.05$ was considered statistically significant. All analyses were performed with SAS software, version 6.12.

RESULTS

A total of 154 patients participated in the study from January 2003 to June 2007. No patient withdrew from the trial during 6 mo follow-up. Seventy-one subjects were assigned in group A, whose average tumor size was 4.3 ± 1.1 cm. Eighty-three participants were in group B, whose average tumor size was 4.1 ± 1.0 cm. The characteristics of the two groups are shown in Table 1. There was no significant difference between the two groups in patients' clinical profile except for the gender ratio.

Table 1 Pre-RFA clinical profile of patients (M_0N_0) *n* (%)

	Group A (*n* = 71)	Group B (*n* = 83)	*P* value
Age (yr)	59.3 ± 12.0	61.3 ± 12.0	0.28
HCC size (cm)	4.3 ± 1.1	4.1 ± 1.0	0.21
Male	63 (88.73)	61 (73.49)	0.02
Cirrhosis	53 (74.65)	72 (86.75)	0.06
Previous surgery	18 (25.35)	14 (16.87)	0.20
Liver disease history			
Hepatitis B	54 (76.05)	72 (86.75)	0.06
Hepatitis C	6 (8.45)	1 (1.20)	
Alcohol-related hepatitis	2 (2.82)	0 (0)	
No	9 (12.68)	10 (12.05)	
Child-Pugh class			
A	58 (81.69)	61 (73.49)	0.23
B	13 (18.31)	22 (26.51)	
TNM staging			
T_1	32 (45.07)	37 (44.58)	0.27
T_2	19 (26.76)	23 (27.71)	
T_3	16 (22.54)	18 (21.69)	
T_4	4 (5.63)	5 (6.02)	

For patients in group A, the feeding artery was blocked in 66/75 (88%) HCC lesions, and decreased in size in 9/75 (12%) lesions. The average number of punctures per HCC was 2.76 ± 1.12. Complete necrosis rate at 1 mo after RFA was 90.67% (68/75), and recurrence rate at 6 mo was 17.33% (13/75, lesions) (Figure 2). For patients in group B, the average number of punctures per HCC was 3.36 ± 1.60. Complete necrosis rate at 1 mo after RFA was 90.20% (92/102), and recurrence rate at 6 mo was 31.37% (32/102).

No significant difference was found in the necrosis rate at 1 mo post-RFA. However, there was a significant different between the two groups in the average number of punctures per HCC and recurrence rate at 6 mo after RFA. The recurrence time between the two groups was also significantly different by the log-rank test ($\chi^2 = 5.23$, $P = 0.02$; Figure 3). When adjusted for gender, age and cirrhosis, it was still significantly different ($\chi^2 = 4.58$, $P = 0.03$).

In group A, RFA-related major complications were seen in five patients (7.04%), including three cases of pleural fluid collection, one of bowel wall edema, and one of intrahepatic biliary duct dilation with jaundice, which were all relieved by conservative therapy. A small amount of subcapsular hemorrhage around the puncture site, approximately 0.5-1 cm thick, during RFA was seen with US in 13 patients (18.31%) (Figure 4). It was not considered as a major complication because homeostasis was achieved after injection of hemocoagulase, without additional intervention and no change in blood pressure. In group B, major complications were detected in nine cases (10.84%), including one of pneumothorax with pleural fluid collection, six of pleural effusion, one of abdominal wall abscess, and one of intraperitoneal hemorrhage in a tumor > 5 cm and protruding liver lateral surface. All complications were controlled

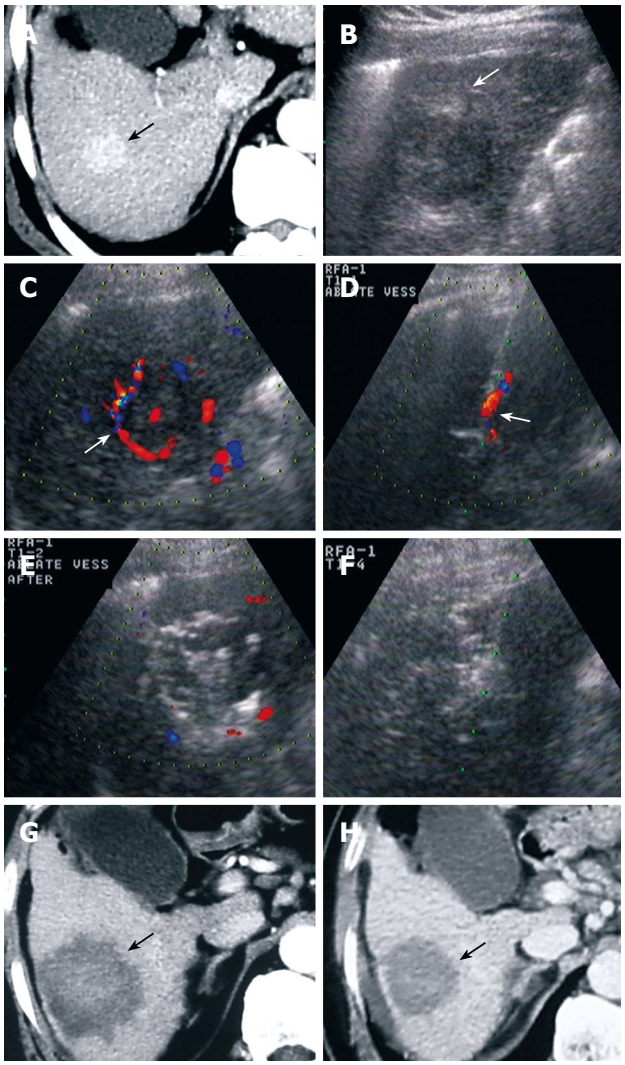

Figure 2 A 65-year-old man with cirrhosis and Child-Pugh class A liver function. An HCC lesion was diagnosed during regular US examination. A: CT showed a tumor with a size of 3.2 cm × 3.0 cm in the right liver lobe; B: US showed a tumor (arrow) of 4.3 cm × 3.3 cm 3 mo later; C: CDFI showed blood flow into and around the tumor with a velocity of 56.7 cm/s; D: CDFI-guided PAA at the area where the feeding artery entered the tumor (arrow), to block the tumor blood supply; E: After PAA, CDFI showed that the previous feeding artery disappeared and no flow signal within HCC; F: After PAA, RFA was performed in the rest of the tumor; G: Contrast-enhanced CT (1 mo after treatment) showed an ablated area covering the previous tumor, without enhancement; H: Contrast CT (6 mo after treatment) showed no enhancement of the ablated area. The patient has survived more than 10 mo without tumor.

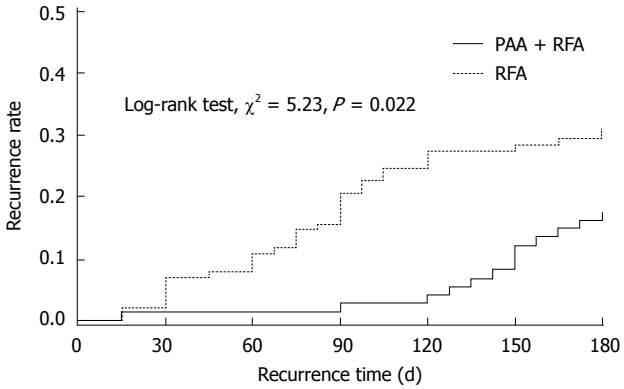

Figure 3 The Kaplan-Meier curves for 6-mo recurrence rate in the two groups.

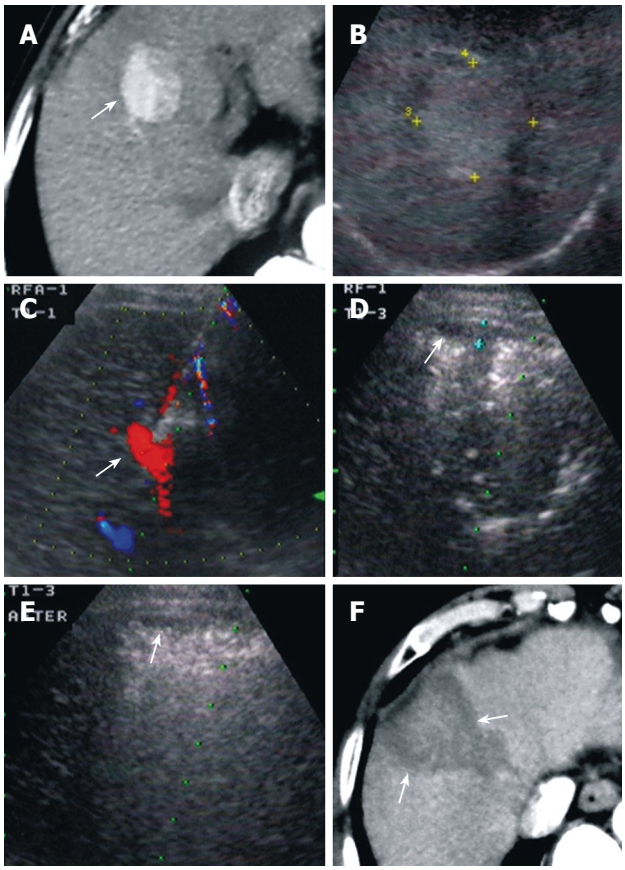

Figure 4 A 72-year-old man with 10 years of hepatitis B was detected with HCC during routine examination. This patient could not tolerate most of the therapies because of old age and poor general condition. A: Contrast-enhanced CT showed a 3.7 cm × 3.5 cm mass and the feeding vessel (arrow); B: Contrast-enhanced US 2 mo later showed the tumor enlarged to a size of 5.2 cm × 4.2 cm; C: CDFI showed feeding vessels in and around the tumor with a flow velocity of 34.5 cm/s. PAA was performed under CDFI guidance; D: During PAA, a small amount of subcapsular hemorrhage (0.5 cm in depth) was noted; E: The amount of hemorrhage gradually reduced (arrow) after PAA finished; F: Contrast-enhanced CT (24 h after treatment) showed a necrotic area covering the tumor. A small amount of subcapsular hemorrhage was still be noted.

with conservative treatment. There was no significant difference between the incidence of complications between the two groups.

DISCUSSION

The outcomes of RFA for HCC correlate closely with the location and blood supply of the tumor. Goldberg *et al*[10] have demonstrated that blood-flow-induced thermal loss in the tumor and liver tissue is the main reason for the decreased ablation effect of thermotherapy. The high-velocity blood flow of the tumor vessels created a heat sink effect that compromised the ablation effect, which led to residual and recurrent HCC. For the treatment of hypervascular HCC, many studies have focused on reducing flow perfusion to improve the thermal effect of RFA. Curley *et al*[14] have used the Pringle method[15] intraoperatively to reduce liver blood flow, by temporarily stopping portal vein and hepatic artery

flow, and improving ablation outcomes. Goldberg *et al*[16] have used vascular agents, such as halothane, vasopressin and adrenaline, to adjust liver blood volume in order to increase ablation area.

TACE is one of the major interventional methods for HCC treatment, and when performed before RFA, it can increase therapeutic efficacy as a result of the decreased heat sink effect[17-19]. Kitamoto *et al*[20] have compared the therapeutic effects of RFA alone and in combination with TACE in 21 patients with 26 HCCs smaller than 3.0 cm. The size of the ablated necrotic area in the TACE/RFA group was significantly larger than that with RFA alone. However, repeated TACE treatment worsened liver function and quality of life[21], and then prolonged the interval between TACE and RFA. Therefore, for those who were ineligible or could not tolerate TACE treatment, other minimally invasive methods of reducing tumor blood supply before RFA were needed.

The study used PAA to ablate the area where the feeding artery entered the tumor, with small overlapping, high-energy ablation foci. This procedure was conducted through one puncture point using three ablations in different directions or depths. After PAA, the tumor's feeding artery was blocked, thus reducing the blood-flow-induced heat loss, and achieving a similar result to that with TACE before RFA. PAA avoided damage to the surrounding liver parenchyma and liver function compared with TACE, and was well-tolerated by patients.

Recurrence after RFA remains an unsolved problem for large HCC. Harrison *et al*[22] reported percutaneous RFA in 46 HCC patients within 3 years, and only 14 (28%) of them showed no liver tumor tissue by imaging and AFP follow-up. Ruzzenete *et al*[23] have reviewed RFA of 104 HCCs in 88 patients with an average tumor size of 3.9 ± 1.3 cm. The necrotic rate for tumors < 3 cm, 3-5 cm and > 5 cm was 100%, 87.7% and 57.1%, respectively. In an average 19.2-mo follow-up period, 17 (19.3%) patients showed local recurrence. In our study, all the HCCs were > 3 cm in both groups A. For HCC treated with PAA and RFA in group A, the local recurrence rate at 6 mo was 17.33% (13/75), which was significantly lower than that in group B (31.37%, 32/102), which was treated with RFA alone ($P = 0.0382$). Although the proportion of cirrhosis was higher in group B than in group A, which might have influenced HCC recurrence, after adjusting for cirrhosis, the results still showed a significant difference between the two groups for recurrence time. Thus, PAA that was performed before routine RFA improved the treatment efficacy in hypervascular HCC. With the new strategy of PAA combined with RFA, the number of percutaneous punctures per HCC was reduced in group A to an average of 2.76 ± 1.12, which was significantly less than that in group B, 3.36 ± 1.60 ($P = 0.001$), thus injury to patients was reduced. Kitamoto *et al*[20] reported that the average duration between TACE and RFA was 18.2 d. In our study, RFA could be performed immediately after blocking of the major feeding artery with PAA, which could reduce hospital stay.

In group A, 13 (17.33%) patients had a small amount of bleeding during PAA, most of which was detected at the first puncture, where the RFA needle punctured the area where the feeding artery entered the tumor. We supposed that the bleeding might have been caused by damage of the feeding vessels and incomplete ablation. Additional focal ablation in the area was helpful in stopping bleeding.

One limitation of our study was the short duration of follow-up. However, our main goal was to demonstrate the benefit of PAA/RFA in treating hypervascular HCC, and our data confirmed this. PAA blocked the feeding artery of the tumor, and then blood-flow-induced heat loss was reduced during RFA treatment. We think that the short-term benefits of PAA/RFA compared with RFA alone for hypervascular HCC provide some insight for the future wider application of this treatment.

In conclusion, for hypervascular HCC patients who were unsuitable for surgical resection or TACE, PAA was an alternative for blocking the feeding artery of the tumor, and reducing heat loss during subsequent RFA. The combination of PAA and RFA could significantly decrease post-RFA recurrence and provide a safe and effective treatment for hypervascular HCC.

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COMMENTS

Background

Hepatocellular carcinoma (HCC) is the most common primary malignant liver neoplasm worldwide. Although surgical resection is the gold standard for treatment of HCC, only a limited number of patients are surgical candidates because of their lack of hepatic reserve that results from coexisting advanced cirrhosis, widespread intrahepatic involvement, and concomitant diseases. Therefore, a variety of imaging-guided tumor ablation therapies such as ethanol injection, microwave coagulation, percutaneous radiofrequency ablation (RFA) and laser ablation are often considered as alternative options. Among them, RFA has been used increasingly as a safe technique for treating hepatic tumors.

Research frontiers

For hypervascular HCC, RFA appears less effective because of the blood-flow-induced heat sink effect, which might cause incomplete ablation or recurrence. Previous experiments have shown that mechanical and pharmacological strategies that are aimed at lowering hepatic perfusion can increase the size of thermally induced lesions.

Innovations and breakthroughs

Repeated transcatheter arterial chemoembolization (TACE) worsened liver function and quality of life, and then prolonged the interval between TACE and RFA. In order to overcome these disadvantages, the study used PAA to ablate the area where the feeding artery entered the tumor, with small overlapping, high-energy ablating foci. This procedure was conducted through one puncture point using three ablations in different directions or depths. After PAA, the tumor's feeding artery was blocked, thus reducing the blood-flow-induced heat loss, and achieving a similar result as that with TACE before RFA. PAA avoided damage to the surrounding liver parenchyma and liver function compared with TACE, and was well-tolerated by patients.

Applications

The study results suggested that, for hypervascular HCC patients who were unsuitable for surgical resection or TACE, PAA was an alternative for

effectively blocking the feeding artery of the tumor, and reducing heat loss during subsequent RFA. The combination of PAA and RFA may significantly decrease post-RFA recurrence and provide a safe and effective treatment for hypervascular HCC.

Terminology

PAA: Color Doppler flow imaging was used to identify the major feeding artery and guide the RFA needle to puncture the area where the feeding artery entered the tumor. This area was ablated with 2-3 overlapping high-energy ablation foci (2-3 cm each in diameter) in different directions or depths.

Peer review

This is a good original study in which authors performed a new approach to block the major feeding artery and reduce heat loss during subsequent RFA. The results are interesting and suggest that the combination of PAA and RFA significantly decreases post-RFA recurrence and provides a safe and effective treatment for hypervascular HCC.

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BRIEF ARTICLES

Comparison of patients by family history with gastric and non-gastric cancer

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Abstract

AIM: To compare the gastric cancer (GC) patients by their family history with gastric and non-GC.

METHODS: Positive family histories within second-degree relatives and clinicopathological features were obtained for 256 patients.

RESULTS: Of the 256 probands, 112 (76 male, 36 female) were incorporated into familial GC (FGC) group: at least two GC members; 144 (98 male, 46 female) were included in the non-FGC group (relatives only affected with non-GCs). Of 399 tumors in relatives (181 from FGC against 212 from non-FGC), GC was the most frequent, followed by esophageal, hepatocellular, and colorectal cancer. Nasopharyngeal cancer was next to lung cancer but prior to breast and urogenital cancers. Most affected members aggregated within first-degree relatives (FGC: 66 siblings, 48 fathers, 31 mothers, four offspring; non-FGC: 56 fathers, 55 siblings, 43 mothers, and 15 offspring). The ratio of males to females in affected first-degree relatives was usually higher in male probands. Paternal history of GC was a slight risk for GC in males (OR = 1.19, 95% CI: 0.53-2.69), while risk of GC by maternal history of non-GCs was increased in females (OR = 0.46, 95% CI: 0.22-0.97). Diffuse-GC was the major histological type in all subgroups. Difference in tumor sites between the two groups was derived from an excess of upper sites in non-FGC female probands.

CONCLUSION: Distribution of associated non-GCs in a family history of GC may vary with geographic areas. GC may have different genetic and/or environmental etiology in different families, and a certain subtype may be inherited in a female-influenced fashion.

Key words: Gastric cancer; Family history; Familial gastric cancer; Familial predisposition; Female-influenced fashion

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INTRODUCTION

It has long been recognized that approximately 10% of gastric cancer (GC) patients present with some kinds of familial aggregation[1]. Family history of GC has previously been studied in many regions, including eastern Asian[2-9], North American[10-12], northern European[13-15], and Mediterranean countries[16-21]. A much higher incidence of familial GC (FGC) was reported from Mediterranean countries[19], while a relatively low occurrence was noted in northern European countries[14]. Some studies, although not all, showed that a family history of GC might be considered a stronger risk factor for women[2,3], or that risk of GC might be higher for subjects with an affected sibling rather than a parent[13,15,16,21]. The risk of GC associated with family history for non-GC has been found with different cancers in different studies[5,10,15,21]. In addition, the histological type of FGC was pronounced for "intestinal" and/or "diffuse" cancers in different studies[13,16]. An unusual form, the hereditary diffuse GC, which is a typical case of FGC caused by a truncation

germline mutation of CDH1, has never been found in eastern Asia[8]. Therefore, a number of features of FGC, including incidence, clinicopathological characteristics, family member risk and etiology, are still in debate.

Previous studies have indicated that familial predisposition to GC may have different genetic and/or environmental correlations in different populations. Although GC is one of the most common cancers in China, few data about its family history are available. Thus, to provide further data on the issue, we designed a proband-based case-control study to explore the traits related to family history of GC in south China.

MATERIALS AND METHODS

The data were obtained from three hospitals in Guangdong Province in south China: the First Affiliated Hospital of Sun Yat-Sen University, the First Affiliated Hospital of Guangdong Pharmaceutic University, and the People's Hospital of Meizhou city. A total of 2260 patients with histologically demonstrated GC admitted between 2000 and 2007 were enrolled in this study. A patient with positive oncologic family history records during his hospitalization or the follow-up was identified as a proband. Features of a proband, including gender, age of onset, and site and histological type of tumor, were taken into account. In addition, the oncologic family history of a proband consisting of data on gastric and/or non-GCs in the relatives was evaluated.

The oncologic family history of each proband was obtained using structured interviews carried out either in person or by telephone. The interviews were always conducted by the same two medical clinicians. After a brief explanation of the purpose of the study, verbal consent was requested before starting the questionnaire. The patient or the closest relative, usually a wife, husband or child, was asked to report the family history. Since an accurate history of third-degree relatives of a proband was relatively hard to obtain, we focused on the first to second-degree relatives, asking about the total number of family members, whether anyone had been diagnosed with cancer, the type of the cancer, and the age of onset.

In this study, according to present recognition[1], a positive family history was defined as a history of cancer within second-degree relatives, while FGC was referred to the presence of at least two GC family members, but also included relatives with history of non-GC. A clear positive family history was obtained for 256 of the total patients. The 256 probands were divided into two groups: a FGC group; and a non-FGC group (patients with relatives affected with non-GCs only), then each group was sorted again according to gender. Tumor location of a proband was identified by three sub-types: upper, medium, and lower site. Histological typing, usually made according to WHO classification, was converted to a Lauren classification. Chi-square test and risk estimate (OR) were used to evaluate the statistical significance. A P value less than 0.05 was considered statistically significant. All statistical analyses were performed using the SPSS 13.0 software.

RESULTS

Of the 256 probands, 112 subjects (76 male, 36 female) reported a family history of GC (it also included relatives with histories of non-GC) and were incorporated into the FGC group; 144 subjects (98 male, 46 female) reported only a family history for non-GC and were included in the non-FGC group. Mean age of probands in FGC and non-FGC were 56.7 years (range 20-87 years) and 55.4 years (range 28-77 years), and the proportions of young patients (no more than 45 years) were 18.8% (21/112) and 20.1% (29/144), respectively ($\chi^2 = 0.08$, $P > 0.05$).

Table 1 shows the overall ranking of associated tumors within second-degree relatives of the 256 probands. A total of 399 neoplastic diseases were reported, 182 from FGC compared with 217 from non-FGC. GC was by far the predominant tumor in affected relatives of FGC (154 GCs against 28 non-GCs) as well as in the overall affected relatives of both groups. Of the non-GCs from affected relatives, esophageal cancer was the most frequent, hepatocellular cancer was the second, and colorectal cancer was the third. The "Canton tumor", a nasopharyngeal cancer (NPC) that has been rarely reported in previous studies, was the fifth, after lung cancer, but prior to breast and urogenital cancers.

Table 2 presents the distribution and number of affected family relatives of the 256 probands. Most affected members aggregated within the first-degree relatives. Among the total 318 (195 male, 123 female) affected first-degree relatives, males were more predominant in members affected with GCs (90 male *versus* 39 female) than in those with non-GCs (105 male *versus* 84 female) ($\chi^2 = 6.53$, $P < 0.05$). In general, among the first-degree relatives, siblings were the most frequently affected relatives, fathers were the next, and mothers were the third. This sequence was repeated among the relatives of FGC (siblings 66, fathers 48, mothers 31), but affected fathers were the most frequent in non-FGC (fathers 56, siblings 55, mothers 43). The ratio of males to females in affected first-degree relatives was usually higher in male than in female probands, and showed a decreasing trend in the four subgroups (Table 3).

Table 4 shows the OR, as estimators of relative risks, together with the corresponding 95% CI for reported parental history of cancers when male and female probands were compared. Paternal history of GC showed a slightly higher risk for males than for females, although there was no statistical significance (OR = 1.19, 95% CI: 0.53-2.69). In contrast, maternal history seemed to affect both genders to the same degree (OR = 0.93, 95% CI: 0.37-2.35). GC risk by paternal history of non-GC was almost the same for both genders (OR = 0.99, 95% CI: 0.48-2.02), but maternal history was less likely to be a risk factor for males, but was a risk factor for females (OR = 0.46, 95% CI: 0.22-0.97). Table 5 exhibits 14 probands whose parents were both affected. Five pairs of affected parents who reported at least one

Table 1 Ranking of associated tumors in family relatives of 256 probands

Associated tumors	FGC (n = 112)		Non-FGC (n = 144)		No. of associated tumors	Percentage
	Male proband[1] (n = 76)	Female proband (n = 36)	Male proband[2] (n = 98)	Female proband[3] (n = 46)		
Stomach	108	46			154	38.6
Esophagus	7	1	43	22	73	18.3
Liver	2	1	17	18	38	9.5
Colorectum	1		23	10	34	8.5
Lung	4	2	10	9	25	6.3
Nasopharynx			14	3	17	4.3
Breast	3		5	5	13	3.3
Urogenital organ		1	4	7	12	3.0
Larynx			5	1	6	1.5
Pancreas			3	1	4	1.0
Others[4]	4	2	12	5	23	5.8
Total	129	53	136	81	399	100

[1]One proband associated with lung cancer; [2]One proband associated with colorectal cancer; another nasopharyngeal cancer. One relative (father) associated with both lung and colorectal cancer; [3]One proband associated with breast cancer; another endometrial cancer; [4]Other malignant tumors including brain tumors, leukemia, lymphoma, oral cancer, and thyroid cancer.

Table 2 Distribution and number of affected relatives of 256 probands

Affected relatives	FGC (n = 112)				Non-FGC (n = 144)		No. of affected relatives	Percentage
	Male proband (n = 76)		Female proband (n = 36)		Male proband (n = 98)	Female proband (n = 46)		
	GC	Non-GC	GC	Non-GC	Non-GC	Non-GC		
Father	31	3	14		38	18	104	26.5
Mother	17	5	9		24	19	74	18.8
Brother	34	4	10	2	23	7	80	20.4
Sister	7	2	5	2	16	9	41	10.4
Son	1				3	7	11	2.8
Daughter	1	1		1	2	3	8	2.0
Second-degree	17	5	8	2	27	16	75	19.1
Total	108	20	46	7	133	79	393	100

Table 3 Ratio of male to female in affected first-degree relatives in four subgroups

Gender of affected relatives	FGC (n = 112)		Non-FGC (n = 144)	
	Male proband (n = 76)	Female proband (n = 36)	Male proband (n = 98)	Female proband (n = 46)
Male	73	26	64	32
Female	33	17	42	31
Total	106	43	106	63
Ratio of male to female	2.21	1.53	1.52	1.03

$\chi^2_{trend} = 5.03$, $P < 0.05$.

suffering from GC had five affected sons only, while nine pairs of parents both suffering from non-GCs had five affected daughters and four affected sons.

Table 6 displays the histological types of the 256 probands. According to Lauren classification, diffuse GC was the major histological type in all subgroups. The frequency of the intestinal type was lower in the FGC than in the non-FGC group, but no statistical difference was found between them. Table 7 shows the distribution of tumor sites of the 256 probands. The lower site was the most frequent tumor location in FGC probands, in contrast to upper sites in non-FGC probands ($\chi^2 = 10.69$, $P < 0.05$). The statistical difference in tumor sites between the two groups was derived from an excess of upper sites presenting in non-FGC female probands (male subgroups: $\chi^2 = 4.99$, $P > 0.05$; female subgroups: $\chi^2 = 9.67$, $P < 0.05$).

DISCUSSION

In the present study of family history of GC in south China, we compared the association between the family history with GCs and non-GCs, and the risk of GC between male and female probands by parental oncologic history. Our study confirmed that overall ranking of associated non-GCs in relatives was different from that reported in other studies to some degree, and that the ratio of males to females in affected first-degree relatives was usually higher in male than in female probands. We also found that the risk of GC was increased in females

Table 4 Relative risk of GC by comparing male with female probands from their paternal or maternal history of cancers

Affected parents	FGC[1] (*n* = 108)		OR (95% CI)	Non-FGC (*n* = 144)		OR (95% CI)
	Male proband (*n* = 72)	Female proband (*n* = 36)		Male proband (*n* = 98)	Female proband (*n* = 46)	
Father	31	14	1.19 (0.53-2.69)	38	18	0.99 (0.48-2.02)
Mother	17	9	0.93 (0.37-2.35)	24	19	0.46 (0.22-0.97)

[1]Deletion of four male probands with parental history of non-GCs.

Table 5 Comparison of 14 probands with both affected parents

Serial number	Father	Mother	Proband	Adenocarcinoma of proband
1	GC	GC	Son	Poorly
2	GC	BC	Son	Poorly
3	GC	EMC	Son	Poorly
4	GC	EC	Son	Poorly
5	HC	GC	Son	Poorly
6	HC	EC	Daughter	Moderately
7	EC	EC	Son	Moderately
8	EC	EC	Daughter	Moderately
9	CRC	EC	Son	Poorly
10	CRC	BC	Daughter	Poorly
11	LC	CRC	Son	Poorly
12	LC	LC	Daughter	Mucinous
13	NPC	PC	Son	Poorly
14	NPC	HC	Daughter	Signet-ring cell

BC: Breast cancer; CRC: Colorectal cancer; EC: Esophageal cancer; EMC: Endometrial cancer; GC: Gastric cancer; HC: Hepatocellular cancer; LC: Lung cancer liver; NPC: Nasopharyngeal cancer; PC: Pancreatic cancer; Moderately: Moderately differentiated adenocarcinoma; Poorly: Poorly differentiated adenocarcinoma; Mucinous: Mucinous adenocarcinoma; Signet-ring cell: Signet-ring cell carcinoma.

when a maternal history of non-GC was present. On the other hand, the risk for GC in males was only slightly (not significantly) increased if a paternal history of GC was present. Moreover, a trend toward upper tumor sites was observed only in females with a family history of non-GC.

Associated tumor categories and their frequencies of family history of GC have been reported previously. Recent studies were consistent with the higher predisposition to gastric than to any of the non-GCs in family members[2,19]. The frequency of GC among all affected relatives was also highest in our study. However, the frequency of non-GC in family members differed from the results in other reports. For example, colorectal, lung, and uterine cancers were the highest in ranking in a Japanese report[9], while colorectal, breast, and lung cancer were prevalent in an Italian report[20], lung/larynx cancer, gastrointestinal cancer, and leukemia/lymphoma were most frequent in a study from Turkey[18], and colorectal and lung cancer were most common in a report from Taiwan[6]. In general, the overall ranking of associated non-GCs in family histories of GC varied from region to region, but the preceding tumors were usually the most frequent ones occurring in that general population.

Eastern Guangdong is one of the regions with the highest incidence of esophageal cancer in China. Guangdong is also one of the regions with the highest incidence of hepatitis B, and HBV-related hepatic cancer is one of the most common tumors in this region. Therefore, esophageal and hepatic cancers, which are usually frequent in general population, are more common in Guangdong. This may be interpreted as that esophageal and hepatic cancers were the two most frequently associated non-GCs among the relatives in our study. Moreover, Guangdong is the region with the highest incidence of NPC in the world. NPC, rare in other general populations, is quite common among Cantonese. This may explain why NPC was much more frequently reported in our study in contrast to other studies, but was less frequently encountered with the most common tumors (usually common both in local residents and in the general population) within our study groups. From this, we can infer that the overall rankings of associated non-GCs in family history of GC may be correlated to the categories of common cancers in the general population, but also show their own regional incidences. This suggests that GC probably shares similar genetic and/or environmental etiologic pathways with other common tumors.

The risk of GC by family oncologic history has been debated in different studies. Although no normal control was included as part of this study, we assumed that siblings being the most frequently affected relatives might support the idea that the risk of GC was higher for subjects with an affected sibling rather than an affected parent[13,15,16,21]. It may be also debated if the risk of GC has no association with family history of any cancer other than GC, because our data showed a very high proportion of relatives affected with esophageal cancers, and esophageal cancer usually shares very similar geographic distribution with gastric cardia cancer in China[22].

Gender-influenced familial predisposition to GC has also been investigated previously[2,3]. Although our study was not a population-based case-control study, the results reconciled a number of points raised in a Japanese study[2]. The findings of this study indicated that risk of GC by paternal history of GC seemed to be a slight risk for males compared with females, while the increment in the risk for GC was prominent in females when they reported a maternal history of non-GCs. However, we did not find a higher risk for females when they reported a maternal history of GCs, which had been described in the previous study[2].

Besides gender-related familial risk of GC, a gender difference was also found in affected first-degree

Table 6 Distribution of tumor histological types (converting the WHO to the Lauren classification) of 256 probands

Lauren classification	WHO classification	FGC (n = 112)		Non-FGC (n = 144)	
		Male proband (n = 76)	Female proband (n = 36)	Male proband (n = 98)	Female proband (n = 46)
Intestinal type	Well	3		2	
	Moderately	19	6	39	12
	Mucinous	5	3	5	3
	Total	27	9	46	15
Diffuse type	Poorly	44	24	39	25
	Signet-ring cell	3	2	11	6
	Undifferentiated	2	1	2	
	Total	49	27	52	31

Well: Well differentiated adenocarcinoma; Undifferentiated: Undifferentiated carcinoma; No statistical differences of histological types were found between any two subgroups, although ratio of intestinal to diffuse types was lower in FGC than in Non-FGC (36/76 = 0.47 from FGC against 61/83 = 0.73 from non-FGC).

Table 7 Distribution of anatomical sites of tumors in 256 probands

Tumor site	FGC (n = 112)		Non-FGC (n = 144)	
	Male proband (n = 76)	Female proband (n = 36)	Male proband (n = 98)	Female proband (n = 46)
Upper	22	5	42	16
	27		58	
Medium	20	7	27	15
	27		42	
Lower	34	24	29	15
	58		44	
χ^2, P value	$\chi^2 = 10.69$, $P < 0.05$			

Difference of tumor sites between two male subgroups: $\chi^2 = 4.99$, $P > 0.05$; two female subgroups: $\chi^2 = 9.67$, $P < 0.05$.

relatives in different families in our study. The ratio of males to females in the affected first-degree relatives was usually higher in FGC than in non-FGC. This may be attributed to the male predominance in GC development. However, the ratio of the affected relatives was usually higher in male than in female probands in both groups. This may also indicate that gender influences the familial predisposition to GC in a certain way. Moreover, gender variations still existed in the affected offspring when both parents suffered from different cancers. Affected daughters were not found in families in which at least one of the two affected parents had GC, but were more frequent in families in which both affected parents suffered from non-GCs. These differences in GC risk by gender and family oncologic history imply that familial predisposition to GC may have a compound genetic and/or environmental correlation in different families.

Lauren's classification system classifies GC under two major histological variants: an intestinal type, likely to be related to environmental factors, and the diffuse type, more likely to have a primary genetic etiology. Our data show that the diffuse type was the more common histological form in all subgroups. However, the frequency of histological type of GC with oncologic family history varied with different studies. Japanese studies[4,7] reported that the undifferentiated histological type was dominant in FGC, and there was a predisposition to the intestinal type when both parents suffered from GCs while to the diffuse type when both parents suffered from non-GCs. The dominance of the intestinal type and the diffuse type was reported in Italy and Poland, respectively[13,16,20]. These variations indicate that familial predisposition to GC may be a multifactor disease.

Many studies have verified that environmental factors may play a more important role than do host genetics in GC development. The prevalence of *Helicobacter pylori* infection has been regarded as an important risk factor for familial aggregation of GC, and may be a strong risk factor for distal, rather than for proximal, GC[23,24]. However, this could not be fully interpreted for the familial predisposition to GC in south China, because of a higher proportion of diffuse GC in all subgroups and no statistical difference of tumor site between the two male subgroups. Some studies have found no appreciable interactions between family history of GC and environmental factors, such as lifestyles[25]. Our investigation detected a pedigree with a father and two brothers affected with GCs, but one of the brothers, suffering from GC at the age of 62 years, had been adopted by another family in his childhood. This suggests that FGC may be predominantly the diffuse type and may be accounted by factors other than just environmental exposures.

Furthermore, a Japanese study[26] reported that an increment of an upper tumor was observed only in patients with a maternal history of GC, but our study displayed the same increment only in females with family histories of non-GC. Our result might be partially influenced by a higher incidence of esophageal cancer among the family members suffering from non-GCs, because the higher prevalence of upper tumors may be due to the higher prevalence of esophageal tumors in this region which is usually associated with a high incidence of gastric cardia cancer. Site-specific risk for female GC that was linked to a family history of non-GC, as well as site-specific risk for GC linked to a maternal history of GC from a Japanese study, provides further evidence that a type of familial susceptibility to GC, to some extent, may be dominated in a female-influenced way.

Some explanations should be provided for the data demonstrated in this study. We focused the discussion primarily on the data of first-degree relatives, because reports of family history will always be clearer in those we know better (i.e. first-degree relatives) and will be less clear as we extend to second and third degree relatives. Therefore, the data of first-degree relatives may introduce fewer inherent biases. The percentage of positive oncologic family histories and incidences of FGC (11.2% and 4.9%) are much lower than those cited in the literatures. It is possible that our data may not show substantial percentages because some patients were unwilling to tell their family history, but this limitation was unlikely to contribute substantially to the differences we observed. Genetic factors may in fact be more important in a young GC development, and therefore may be likely associated with familial susceptibility in a young patient[27,28]. However, the percentages of young patients among the total patients (20.0%) and the 256 probands (19.5%) had no statistical difference, and both were higher than 10% in GC population cited in literatures[28]. These data indicated that the proportion of young patients in the GC population was higher in south China, but an association between young individuals and the inheritance of GC in a cancer family was not found. Therefore, the etiology and the terms used to describe familial tendency of a young GC patient should be reconsidered.

In conclusion, the overall ranking of associated non-GCs in the family history of GC may vary with geographic areas. Familial predisposition to GC may be related to compound genetic and/or environmental etiologies; and a certain subtype of GC may be inherited in a female-influenced fashion.

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COMMENTS

Background

Gastric cancer (GC) is a major clinical challenge because of its frequency, poor prognosis and limited treatment options. The etiology of GC is still uncertain, but its familial aggregation in a variable but significant proportion of cases suggests the importance of genetic predisposition. The risk of developing GC is greater in relatives of patients with oncological family history than in relatives of sporadic cancer. Previous studies have indicated that familial predisposition to GC may have different genetic and/or environmental correlations in different populations. Although GC is prevalent in China, scanty information about its family history is available.

Research frontiers

Familial predisposition to GC may be partly due to the fact that relatives tend to be exposed to the same environmental risk factors, but also to inheritable susceptibility. In this field, the research hotspot is how to identify risk relatives, and risk factors (including different prevalence of various susceptibility genes, and the impact of various environmental factors) in a family with disease.

Innovations and breakthroughs

Studies on family history of GC, the association of familial risk of GC with the age of onset GC, with family member gender, or with family history of non-GCs, usually yielded contrasting results. This study believed that the overall ranking of associated non-GCs in the family history of GC may depend on geographical variations; familial predisposition to GC may be related to compound genetic and/or local environmental factors; and a certain subtype of GC may be inherited in a female-influenced fashion.

Applications

The data presented in this article represents important data about familial predisposition to GC with a high prevalence. This will add to the available body of knowledge about GC inheritance and aid in future research into this important disease.

Terminology

Familial GC (FGC) is simply designated as a cancer family with at least two GC members, also including those affected with non-GCs. Among the cases of FGC, several situations can be identified according to the histopathologic type of GC and the number of affected relatives, which encompasses specific syndromes/diseases as follows: hereditary diffuse GC, familial diffuse GC, and familial intestinal GC.

Peer review

Zhou *et al* designed a proband-based study to provide further data on factors of familial predisposition to GC. This paper is interesting and written well.

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Major complications after radiofrequency ablation for liver tumors: Analysis of 255 patients

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Abstract

AIM: To investigate the major complications after radiofrequency ablation (RFA) for the treatment of liver tumors and analyze possible risk factors that precipitate these complications.

METHODS: From March 2001 to April 2008, 255 patients with liver tumors (205 male, 50 female; age range, 18-89 years; mean age, 56.0 years) who received RFA were enrolled in this study. Of these patients, 212 had hepatocellular carcinoma, 39 had metastatic liver tumors and four had cholangiocellular carcinoma. One hundred and forty eight patients had a single tumor, and 107 had multiple tumors. Maximum diameter of the tumors ranged 1.3-20 cm (mean, 5.1 cm). All patients were treated with a cooled-tip perfusion electrode attached to a radiofrequency generator (Radionics, Burlington, MA, USA). RFA was performed *via* the percutaneous approach (n = 257), laparoscopy (n = 7), or open surgical treatment (n = 86). The major complications related to RFA were recorded. The resultant data were analyzed to determine risk factors associated these complications.

RESULTS: Among the 255 patients, 425 liver tumors were treated and 350 RFA sessions were performed. Thirty-seven (10%) major complications were observed which included 13 cases of liver failure, 10 cases of hydrothorax requiring drainage, three cases of tumor seeding, one case of upper gastrointestinal bleeding, one case of intrahepatic abscess, one case of bile duct injury, one case of cardiac arrest, and five cases of hyperglycemia. Seven patients had more than two complications. Liver failure was the most severe complication and was associated with the highest mortality. Eleven patients died due to worsening liver decompensation. Child-Pugh classification (P = 0.001) and choice of approach (P = 0.045) were related to post-treatment liver failure, whereas patient age, tumor size and number were not significant factors precipitating this complication.

CONCLUSION: RFA can be accepted as a relatively safe procedure for the treatment of liver tumors. However, attention should be paid to possible complications even though the incidences of these complications are rare. Careful patient selection and the best approach choice (percutaneous, laparoscopy, or laparotomy) will help to minimize the incidence and morbidity rate of complications which occur after RFA.

Key words: Complication; Hepatocellular carcinoma; Metastatic liver tumor; Radiofrequency ablation; Liver failure

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INTRODUCTION

Hepatocellular carcinoma (HCC) and metastatic liver tumors are the two most common malignant tumors of liver. In most cases hepatectomy is the best curative treatment option[1,2]. However, there are some factors that limit the use of surgical resection. Therefore, alternative techniques such as percutaneous ethanol injection (PEI), microwave therapy, radiofrequency

ablation (RFA), laser therapy, and transarterial chemoembolization (TACE) have been used for the treatment of hepatic malignant tumors[3-5]. Among these techniques, RFA is performed more widely than the others because it results in large coagulated necrosis of the tumor, requires fewer treatment sessions, and achieves higher survival rates[6-8]. Thus RFA would be more beneficial to patients than other non-resectional techniques.

Although RFA is considered a relatively safe and minimally invasive technique, it may induce severe complications. The trade-off between the risks and benefits must be considered. Some major post-RFA complications, such as hepatic failure, intraperitoneal bleeding, hepatic abscess, bile duct injury, tumor seeding, and gastrointestinal perforation have been reported[9-11]. The estimated range of mortality rate is 0.1%-0.5%, while the major complication rate is 2.2%-3.1%[12]. A better understanding of the pertinent complications which may occur post-treatment is the key to successful RFA treatment.

Since 2001, we have used RFA to treat patients with HCC or liver metastases. Despite its promising therapeutic effects, RFA has resulted in a number of major complications. In the present study, major complications resulted from RFA procedures during a 7-year-study in a single center have been retrospectively analyzed, and the possible risk factors precipitating these complications were determined.

MATERIALS AND METHODS

Patients

From March 2001 to April 2008, 255 patients with liver tumors (205 male, 50 female; age range, 18-89 years; mean age, 56.0 years) who received RFA were enrolled in this study. Patient characteristics are described in Table 1. Of these patients, 212 had HCC (including 48 cases of recurrent nodules after hepatectomy and five cases of HCC rupture hemorrhage), 39 had liver metastases and four had cholangiocellular carcinoma. One hundred and seventy five patients had chronic liver diseases, of which 168 patients were hepatitis B virus carriers, five patients were hepatitis C virus carriers, and two patients had schistosomiasis liver cirrhosis. Based on Child-Pugh classification, 231 patients (90.6%) were considered as class A, 22 patients (8.6%) as class B, and two patients (0.8%) as class C. Alpha fetoprotein (AFP) serum assays were performed in all patients: 88 patients had normal AFP levels; 92 patients had levels between 5 and 400 ng/mL; and another 75 patients displayed AFP levels of more than 400 ng/mL.

Before treatment, all patients were examined by abdominal ultrasonography, computed tomography (CT) or magnetic resonance imaging (MRI). The location of tumors was defined according to Couinaud's nomenclature. There were 148 uninodular cases and 107 multinodular cases. In 192 patients, we performed RFA only once. In the remaining patients, RFA was performed twice or more due to hepatic recurrence. There were 425 treated tumor nodules in total. The median diameter of the nodules was 5.1 cm (range, 1.3-20 cm), with 174 three centimeters or smaller, 142 between 3.1 and 5 cm, and 109 larger than 5 cm.

Table 1 Characteristics of patients, tumors and treatment approach (mean ± SD)

Patients and tumor characteristics	Value
No. of patients	255
Median age (yr)	56.0 ± 13.2 (range, 18-89)
Gender (male/female)	205/50
Disease (HCC/MHC/CCC)	212/39/4
Background liver disease (HBV/HCV/ no infection)	168/5/82
Patients with liver cirrhosis *n* (%)	172 (67.5)
Portal vein invasion (no/yes)	25/230
Alphafetoprotein (0-5/5-400/> 400 ng/mL)	88/92/75
Child-Pugh classification (A/B/C)	231/22/2
ALT (U/L)	47.6 ± 2.5
AST (U/L)	54.7 ± 3.0
Total bilirubin (μmol/L)	27.4 ± 7.1
Serum albumin (g/L)	38.9 ± 0.3
Tumor stage	425
Number of tumors (uninodular/multinodular)	148/107
Size of tumor (cm)	5.1 ± 3.4 (range, 1.3- 20)
Diagnosis (histopathologic/clinical)	215/210
Treatment	350
Choice of approach (percutaneous/ laparoscopy/laparotomy)	257/7/86
Combination with TACE or PEI (+/-)	61/194

The diagnosis of liver tumors was based on histopathological findings of specimens (129 patients, 50.6%). The remaining tumors (126 patients, 49.4%) were diagnosed based on typical CT or MRI findings and tumor markers.

All the patients had contraindications to hepatectomy due to advanced age, associated diseases, severe liver dysfunction, inappropriate location of the tumor, or refusal to undergo the surgical procedure. Before performing RFA, all patients were informed and signed the consent forms.

Technique

A cool-tip needle radiofrequency system (Radionics, Burlington, MA, USA) was used in all patients. The system consists of a perfusion pump, electrode pads placed on the patient's skin, and a radiofrequency generator that monitors tissue impedance, electric current, power and temperature. The shaft of the needle has internal channels which allow the needle to be perfused with chilled water to maintain the temperature of the tip below 20℃ to prevent charring around the needle tip. The current is automatically adjusted according to the impedance at the needle tip. Each ablation cycle lasts 12 min. Following ablation, the probe tract is cauterized as the RFA needle is withdrawn. Successful ablation usually increases the temperature of the ablated tissue to 60-80℃. RFA was performed depending on the size and localization of the tumor. A single electrode needle was used for patients with liver tumors smaller than 2 cm.

For patients with large tumors (> 2 cm), we performed overlapping ablations to thoroughly eliminate the tumor. The purpose of the treatment was to achieve destruction of the tumor tissue and the 1 cm margin of parenchyma around the lesion. However, for some patients with large HCCs or portal vein tumor thrombosis, RFA was regarded as a palliative treatment.

RFA was performed *via* the percutaneous approach (n = 257), laparoscopy (n = 7), or by open surgical treatment (n = 86). All the procedures were performed by a surgeon and a radiologist with more than 3 years experience in RFA. Whenever possible, RFA was performed percutaneously. A surgical or laparoscopic approach was adopted to treat patients with tumors located near visceral organs such as the stomach, colon, or gallbladder. When tumors were found near the hepatic hilum, cholecystectomy was performed before RFA. If tumors were located directly below the diaphragm, we used artificial pleural effusion to obtain an image of the whole tumor by percutaneous ultrasonography[13]. Sixty-one patients had undergone combined treatment for liver tumors (PEI or TACE).

The following serologic values including liver function tests and complete blood counts were measured 3 d and 1 wk after treatment. Treatment efficacy was evaluated by contrast-enhanced CT or ultrasonography 4 wk after treatment. Tumors were considered successfully ablated when no region of enhancement was found either in the entire tumor or in a 0.5 cm margin of normal hepatic tissue surrounding the tumor.

Complications

Majors complications were defined as those that delayed hospital discharge, threatened the patient's life, or led to substantial morbidity and disability[14]. These included liver failure, peritoneal hemorrhage, tumor seeding, and collateral thermal damage to adjacent organs. Differentiation among immediate complications (during the maneuver or ⩽ 24 h after the procedure), periprocedural complications (with 30 d), and delayed complications is advised[12]. Complications were identified on the basis of clinical findings, laboratory or imaging examinations during the RFA and post-treatment observation period.

Statistical analysis

Clinical data of all patients were entered prospectively into a computerized database. We analyzed complications individually instead of combining all the data together. The values of the baseline characteristics, which included patient age, disease, Child-Pugh classification (A *vs* B or C), tumor size and number, treatment approach (percutaneous, laparoscopy or laparotomy), mode of RFA (single or overlapping ablation) were assessed by using Logistic regression analysis. $P < 0.05$ in a two-tailed test was considered statistically significant. All data processing and statistical analysis were performed using commercially available software (SPSS for Windows, Version 11.5).

Table 2 Major complications after radiofrequency ablation

Complication	No. of complications	Disease
Hepatic failure	13	HCCs
Thoracic complications	10	8 HCCs, 1 CCC, 1 MHC
Tumor seeding	3	HCCs
Upper gastrointestinal bleeding	1	HCC
Intrahepatic abscess	1	HCC
Bile duct injury	1	HCC
Cardiac complication	1	HCC
Hyperglycemia	5	HCCs

RESULTS

In the 255 patients, 425 liver tumors were treated and 350 RFA sessions were performed (63 patients had more than one session). The mean follow-up period for the entire group was 32.1 mo (range, 2-84 mo). The median overall survival rate was 21.0 mo. Overall cumulative survival rate at 1-, 2-, and 3-year was 63.1%, 43.3%, and 35.7%, respectively. Thirty five (35/350, 10% per session) major complications were found after RFA treatment (Table 2). Seven patients had more than two types of complications and 28 patients (28/225, 12.4% per patient) suffered from major complications which included 13 (3.7%) cases of liver failure, 10 (2.9%) cases of hydrothorax requiring drainage, 3 (0.9%) cases of tumor seeding, 1 (0.3%) case of upper gastrointestinal bleeding, 1 (0.3%) case of intrahepatic abscess, 1 (0.3%) case of bile duct injury, 1 (0.3%) case of cardiac arrest, and 5 (1.4%) cases of hyperglycemia. The complication rate was 4.0%, 0.3% and 5.7% after percutaneous, laparoscopic and intraoperative RFA, respectively. The immediate complications, periprocedural complications and delayed complications were 2.0%, 29.0% and 4.0% among 350 sessions, respectively.

Liver failure was the most severe complication and was associated with the highest mortality. Eleven patients (11/255, 4.3% per patient) died of worsening liver decompensation. The potential risk factors that might contribute to liver failure were analyzed. We found that Child-Pugh classification (P = 0.001) and choice of approach (P = 0.045) were related to post-treatment liver failure, whereas patient age, tumor size and number did not correlate with this complication. This finding suggested that Child-Pugh classification and choice of approach were independent risk factors for liver function impairment after RFA.

We adopted intraoperative RFA to treat a patient with an HCC located in the right posterior segment close to the right portal vein (8 cm in diameter). We performed overlapping RFA to achieve a complete ablation area, and the ablated frequency was 12 times. A bile leakage occurred 1 wk after the procedure and subsequently resulted in liver abscess. The patient presented with local pain and high fever. Sustained antibiotic therapy and percutaneous ultrasound guided drainage were carried out. The patient also had diabetes mellitus which

made his basic condition fairly poor. Despite aggressive supportive care, the patient succumbed to progressive liver failure and died 5.5 mo after RFA.

Tumor seeding was identified in three patients (0.86%) at 2, 4 and 5 mo after RFA. These seeding foci were located in the subcutaneous tissue of the abdominal wall (two patients) and omentum (one patient). They were treated by surgical resection, High Intensity Focused Ultrasound and RFA, respectively. Of these three patients, two had undergone previous biopsy, two had an AFP level higher than 400 ng/mL, and all had a single tumor that was not near the capsular fibrosa ranging 2-5 cm in diameter. None of these factors were found to be significantly associated with tumor seeding ($P = 0.085$, $P = 0.840$, $P = 0.088$, respectively).

Hydrothorax was found in ten patients. In five of these cases, tumors were located at the dome of the liver. In one patient with a single 2.5 cm diameter subcapsular HCC in segment Ⅵ, cardiac arrest occurred during treatment. She was successfully rescued. Other major complications included one case of upper gastrointestinal bleeding and five cases of hyperglycemia. However, these complications did not result in fatal consequences.

DISCUSSION

RFA has gained wide acceptance as a safe alterative to surgery in the management of early HCC and metastatic liver tumors[15,16]. It produces complete necrosis of the tumor and achieves a satisfactory survival rate with low recurrence rate on long-term follow-up. Despite the benefits, RFA entails some risks as revealed by post-RFA complications. Complications such as liver failure, intraperitoneal bleeding, abscess, bile duct injury, tumor seeding are very serious, and can be life threatening[10,17,18]. Being well aware of the complications and the choice of treatment method will lead to a more practical application and enable this procedure to be safer and more effective.

There have been some analyses of post-RFA complications which have involved a large series of investigations[17]. However, the incidence and mortality of post-RFA complications reported in this study were different from previous reports by other groups. This discrepancy may be attributed to the choice of indications and the skills of the operators. In most of these reports, RFA was performed only when the tumor was no greater than 3 cm in diameter and the patient had no portal vein tumor thrombus. However, in our study, patients with advanced HCC also received RFA treatment, which may have resulted in the relatively high incidence of major complications.

RFA is considered an invasive therapy, especially for those patients with insufficient hepatic reserve to tolerate resection. Therefore, the preoperative evaluation of liver parenchymal function after treatment is of great importance. Decompensated baseline function reserve might precipitate transient liver function impairment after RFA and should be closely followed up. Sepsis and liver failure have been reported as the most common causes of death in a multicenter survey[19]. Koda *et al*[20] analyzed the liver laboratory tests and complications after RFA treatment and found that patients with a high pre-treatment Child-Pugh score suffered from long-term deterioration of liver parenchymal function and subsequent serious complications. The conclusion from that study was that patients with a Pugh score ⩾ eight points would not be good candidates for RFA or TAE-RFA. In our study, the most severe complication was liver failure, which resulted in mortality in 11 patients after treatment. Our findings demonstrated that Child-Pugh classification was related to post-treatment liver failure. This result was consistent with the finding that Child B or C was a risk factor for post-treatment liver failure[21]. Therefore these patients may not be appropriate candidates for RFA. The choice of approach was another independent risk factor associated with liver failure. Among the 13 patients who experienced rapid hepatic decompensation, 10 were treated with overlapping ablation *via* an open surgical technique. Recently, intraoperative RFA has been performed as an approach to treat liver tumors, particularly for difficult lesions adjacent to the diaphragm, bowel, or gallbladder. Tepel *et al*[22] pointed out that although intraoperative RFA was a valuable tool in liver surgery, it added invasiveness and technical difficulties to the procedure and might cause severe post-treatment complications[23]. When these findings are combined with the results from our study, it becomes more obvious that patient selection and choice of approach are the two major factors to consider in order to achieve desirable outcomes similar to those *via* percutaneous RFA.

Bile duct injury is an uncommon severe complication with an incidence of 0.1%-1.0%[19,24]. Kim *et al*[25] demonstrated that although bile duct changes were frequent after the RFA of HCC, it was of no clinical significance in most cases. In addition, major complications requiring additional treatment were rare. Cooling of the biliary tract with chilled saline has been used to prevent biliary injury by RFA[26]. Ohnishi *et al*[27] reported that the incidence of biliary injury was significantly reduced in the intraductal chilled saline perfusion (ICSP) group compared to that in the control group. Moreover, liver function in the treated patients was also better preserved in the ICSP group 6 mo after RFA.

We performed overlapping RFA *via* an open surgical approach to treat a patient with a tumor located in the right posterior segment near right portal vein. The patient suffered from bile duct injury combined with other complications including liver failure, hepatic abscess and hyperglycemia. This result was consistent with a previous report[12] where the possibility of bile duct injury was increased if the mass was located in the central portion of the liver and abutted the portal hepatis. Moreover, excessive heating to overcome the "heat sink effect" of hilar large vessels could cause significant damage to the major ducts.

The incidence of needle-track tumor seeding was reported to range from 0%-12.5%[28,29]. Llovet *et al*[28] reported that neoplastic seeding was related to subcapsular location, poor differentiation state of the tumor cells and high AFP levels (more than 100 ng/mL). Livraghi

et al[30] pointed out that RFA with a cooled-tip needle was associated with a low risk of neoplastic seeding, and only previous biopsy was significantly associated with tumor seeding. In our study, we found no risk factors precipitating this complication, even though superficial tumor location, tumor biopsy procedure and AFP level were taken into account. It should be acknowledged that the inability to identify independent predictors of tumor seeding was most likely related to the low number of patients who had this complication. It is thought that intraperitoneal bleeding may drive tumor cells outside the hepatic capsule[28]. In our study, no bleeding occurred after RFA, which might also contribute to the low incidence of tumor seeding, particularly for subcapsular tumors.

Complications arising from RFA can be divided into two general categories: those related to imaging-guided electrode placement and those related to thermal transmission[12]. Most of the complications such as bleeding, abscess, and gastrointestinal perforation might arise due to improper RFA approach and puncture technique. According to our study, several measures to avoid major complications and to achieve satisfactory therapeutic effect should be taken. Firstly, the most effective strategy to minimize complications is careful patient selection. Preoperative evaluation is of great importance. Patients with Child-Pugh classification B or C and tumors close to vital structures require careful consideration. Secondly, the appropriate approach must be chosen according to the patient's condition. RFA may be performed either percutaneously, *via* laparoscopy or laparotomy, by ultrasound guidance. The trade-off between the risks and benefits must be acceptable. Thirdly, because of the potential incidence of post-treatment complications, a physician should know these pertinent complications, detect the complications as early as possible and provide appropriate management.

In conclusion, RFA is effective for patients with liver tumors. However, liver decompensation may rapidly worsen and lead to life-threatening liver failure, especially in patients with Child-Pugh classification B or C. As the procedure can be associated with major complications, a more accurate selection of candidates and approach to RFA treatment is advisable.

COMMENTS

Background

Although surgical therapy for hepatocellular carcinoma is regarded as standard, most patients are not surgical candidates because of poor liver function or other factors. Many methods of local ablation have been developed to control the disease. Among them, radiofrequency ablation (RFA) is one of the most effective. However, some studies have reported severe complications after RFA, such as hepatic failure, tumor seeding and peritoneal hemorrhage.

Innovations and breakthroughs

This study showed that liver failure was the most severe complication after RFA especially in patients with Child-Pugh classification B or C. The authors also suggested that careful patient selection and the best approach choice might help to minimize the incidence and morbidity rate of complications after RFA.

Peer review

The authors showed the major complications after RFA for the treatment of liver tumors and analyzed the possible risk factors. The results of this research might be important for the clinical application of this therapy.

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Stromal cell derived factor-1 enhances bone marrow mononuclear cell migration in mice with acute liver failure

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Abstract

AIM: To evaluate the number of bone marrow mononuclear cells (BMMC) that are migrated to the liver following transplantation of murine BMMC into mice with acute liver injury.

METHODS: BMMC were isolated from the bone marrow of mice in a lymphocyte separation medium and then labeled with PKH26. The labeled cells were subsequently infused into the caudal veins of BALB/c mice with hepatic injury induced by carbon tetrachloride and 2-acetylaminofluorene. Mice in experimental group were treated with stromal cell-derived factor-1 (SDF-1) which was injected intraperitoneally after transplantation of BMMC. Mice in control group were injected intraperitoneally with 0.1 mL of saline (0.9% NaCl) after transplantation of BMMC. After 2 wk, migration of the cells in experimental group was studied by fluorescence microscopy. The expression of proliferating cell nuclear antigen and albumin was quantified with manual methods in both groups. The serum transaminase levels at different time points were compared between the two groups.

RESULTS: The labeled "cells" were found in the portal region and central veins of hepatic lobules. The PKH26-labeled cells appeared at an average frequency of 108 ± 8/high power field in the experiment group and 65 ± 8/high power field in the control group ($P < 0.05$). The total number of positive cells was 29 ± 7/high power field in the experimental group and 13 ± 2/high power field in the control group. The albumin expression level was also higher in the experimental group than in the control group (29 ± 7 *vs* 13 ± 2, $P < 0.05$). The total number of crossing points was 156 ± 5/high power field in the experimental group and 53 ± 5/high power field in the control group ($P < 0.05$). The serum alanine aminotransferase levels in experimental and control groups were measured at different time points (120 ± 40 *vs* 118.50 ± 1.75, $P > 0.05$; 80.60 ± 6.50 *vs* 101.08 ± 5.67, $P < 0.05$; 50.74 ± 5.38 *vs* 80.47 ± 4.62, $P < 0.05$; 30.54 ± 2.70 *vs* 60.72 ± 4.37, $P < 0.05$; 30.77 ± 5.36 *vs* 40.47 ± 6.50, $P < 0.05$). At the same time, the serum aspartate aminotransferase levels were measured in experimental and control groups at different time points (122.55 ± 1.46 *vs* 120.70 ± 4.22, $P > 0.05$; 54.26 ± 6.50 *vs* 98.70 ± 8.20, $P < 0.05$; 39.47 ± 5.39 *vs* 78.34 ± 4.50, $P < 0.05$; 28.94 ± 2.70 *vs* 56.44 ± 4.28, $P < 0.05$; 30.77 ± 5.45 *vs* 42.50 ± 6.28, $P < 0.05$).

CONCLUSION: SDF-1 can promote the migration of BMMC to the liver of mice with acute liver failure.

Key words: Stromal cell derived factor-1; Bone marrow mononuclear cell; Acute liver failure; Transplantation; Mobilization

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INTRODUCTION

Stem cells have the potential ability of multi-directional

differentiation and self-renewal. Under proper induction circumstances, they can differentiate into different functioning cells. Differentiation between groups is ongoing. Recent studies using experimental animal models and samples from clinical mobilization protocols demonstrated that chemokines such as stromal derived factor-1 (SDF-1) and IL-8 are involved in the mobilization process[1,2]. The central role of SDF-1 in induction of mobilization has been reviewed[3]. SDF-1, a kind of micro-molecular proteins, possesses a variety of biologic activities. It has been identified that SDF-1 can promote bone marrow stem cell directional differentiation both in heart tissue[4] and in nerve tissue[5]. Since mesenchymal stem cells in human second-trimester bone marrow, liver, lung, and spleen exhibit a similar immunophenotype but a heterogeneous multilineage differentiation potential l[6], we hypothesize that SDF-1 can also mobilize migration of bone marrow mononuclear cells (BMMC) in mice with acute liver failure.

The aim of this study was to determine whether autologous BMMC can be mobilized by SDF-1 in BALB/c mice with experimental acute liver failure. To test this, BMMC were isolated from the bone marrow of mice in a lymphocyte separation medium and then labeled with the fluorochrome dye PKH26.The labeled cells were subsequently infused into the caudal veins of mice with hepatic injury induced by carbon tetrachloride and 2-acetylaminofluorene. Mice in experimental group were injected intraperitoneally with SDF-1 (5 μg/kg) and mice in control group were injected intraperitoneally with 0.1 mL of saline (0.9% NaCl) after transplantation of BMMC. After 2 wk, the migration of BMMC was studied by fluorescence microscopy. The number of migrated BMMC was calculated, and the expression of proliferating cell nuclear antigen (PCNA) and albumin in both groups was quantified with manual methods. In the following 4 wk, serum aminotransferase activity was detected to monitor the changes in liver function at different time points.

MATERIALS AND METHODS

Experimental animals

Male BALB/c mice, weighing 20-22 g, at the age 8-10 wk, were purchased from the Animal Center of Jilin University. All mice were housed in rooms at a constant temperature and humidity in a 12 h light/dark cycle with free access to normal rodent chow and water. Experiments were conducted according to the guidelines established by Jilin University. The procedures were approved by the Supervisor Committee of Jilin University Animal Council.

Principal reagents

Recombinant murine SDF-1 α (CXCL12, Catalog #: 250-20A) was obtained from PeproTech EC Company (USA). Proliferating cell nuclear antigen, lymphocyte isolation medium (1.077 g/cm^3) and red fluorochrome PKH26GL were purchased from Simga Company (Saint louis, Missouri USA). 2-acetylaminofluorene was purchased from Invitrogen Company (California, USA).

Experimental groups

The animals were divided into donor and recipient groups. The recipient group was further divided into an experiment group and a control group (n = 30).

Methods

Femoral bones were aseptically removed from male BALB/c mice under anesthesia and bone marrow in the medullary cavity was bathed by heparin (50 U/mL) dissolved in normal saline. Bone marrow cells were suspended in a sterilized lymphocyte isolation medium. After dilution with 2 mL phosphate-buffered saline (PBS, 0.01 mol/L, pH = 7.4) at 1:1, the cells were slowly added at a relative matching density of 1.077 g/cm^3 lymphocytes followed by centrifugation at 2000 r/min for 20 min. Cell groups were identified, washed with PBS, and centrifuged at 1200 r/min for 10 min. The top of centrifuge tube was shaken lightly to detach the cell groups. A DMEM/F12 medium was added (15% FBS, 100 000 U/L penicillin, pH = 7.4) to prepare cell suspension at a density of over 5 × 10^8 cells/L. Finally, BMMC were labeled with PKH26 according to its manufacture's instructions. The density of labeled cell suspension was adjusted to 3 × 10^7 cells/mL. BMMC with a viability over 95%, measured by trypan blue exclusion, were used. One million of BMMC were isolated from the donor group, labeled with PKH26 and injected into mice of the experimental group *via* the tail vein. One hour later, mice in the experimental group were injected intraperitoneally with SDF-1 (5 μg/kg). Mice in the control group were injected intraperitoneally with 0.1 mL of saline (0.9% NaCl). The injection of SDF-1 or saline was repeated once a day in the following weeks. Two weeks later, the mice were euthanized by cervical spine dislocation with their livers removed immediately and frozen in liquid nitrogen. The number of migrated BMMC in hepatic tissue stained with hematoxylin-eosin and immunohistochemistry was calculated under a fluorescence microscope. The density of serum aminotransferase activity was detected with an automatic biochemistry analyzer.

Establishment of animal acute liver failure model

Carbon tetrachloride (CCl_4) is widely used to generate an experimental model mimicking acute liver injury caused by toxic substances. Mice in the recipient group were treated with 2-acetaminofluorene dissolved in liquid macrogol (20 mg/kg) by lavage, once a day for 7 d. On day 8, the animals were injected intraperitoneally with 20% CCl_4 (0.5 mL/kg, dissolved in vegetable oil). On day 9, one million of BMMC were transplanted into mice *via* the tail vein. From day 10, mice in the recipient group were given 2-acetaminofluorene dissolved in liquid macrogol (20 mg/kg), once a day for 7 d. On day 21, mice in the experimental group were euthanized by cervical spine dislocation. Liver tissue was resected immediately and frozen in liquid nitro-

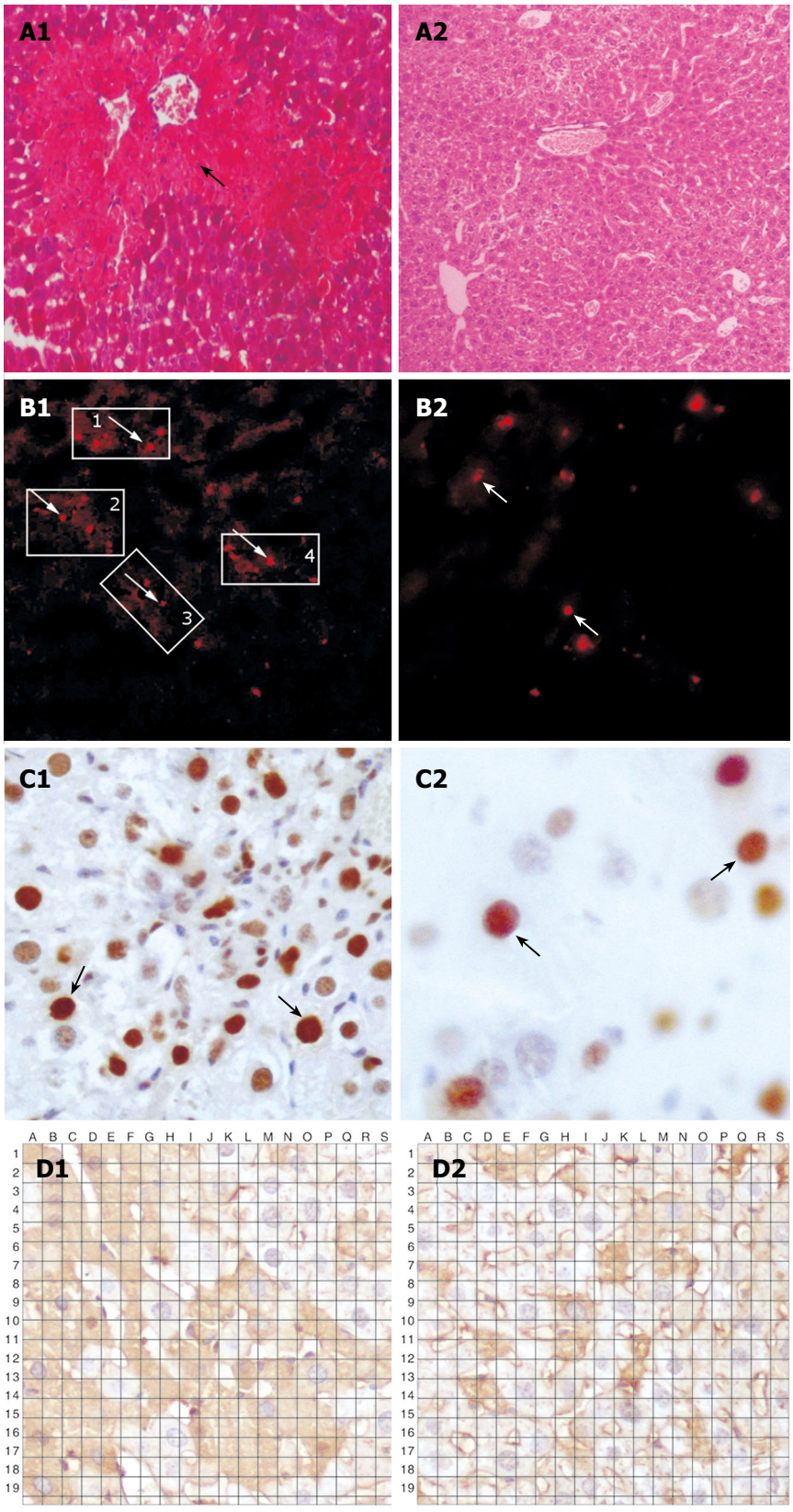

Figure 1 Histopathology of hepatic tissue from the two groups. A: PKH26-labeled cells detected after establishment of acute liver failure animal model with extensive vacuolar degeneration and edema of hepatocytes in acute liver failure (A1) and normal liver tissue (A2); B: Sporadic PKH26-labeled bone marrow stem cells in experimental group (B1) and control group (B2); C: Expression of PCNA in sporadic PKH26-labeled bone marrow stem cells in experimental group (C1) and control group (C2); D: Expression of albumin and sporadic PKH26-labeled bone marrow stem cells in experimental group (D1) and control group (D2) (× 200).

gen. Liver injury was confirmed by fluorescence microscopy and hematoxylin-eosin staining. Extensive vacuolar degeneration and edema of hepatocytes were found in liver tissue (Figure 1A).

Detection index

The number of fluorescence PKH26-labeled cells in liver sections stained with immunohostochemistry (FITC) was counted. Albumin and PCNA in liver tissue sections stained with FITC were also calculated. The density of serum aminotransferase activity was detected with an automatic biochemistry analyzer which can test the liver function at different time points following the transplantation of BMMC.

Tissue preparation

Livers were perfused with 4% paraformaldehyde in 0.01 mol/L phosphate-buffered saline (pH 7.4, PBS) following anesthesia with sodium pentobarbital 100 mg/kg (ip), fixed overnight and cryoprotected in 30% sucrose at 4℃. Liver tissue was cut into 6-μm thick sections.

Immunohistochemistry

Immunofluorescence was carried out by incubating the sections in PBS containing 5% donkey serum and primary antibody-rabbit anti-mouse serum albumin(1:1000, ab19196, abcam, MA, USA), followed by a 2-h reaction with Alexa Fluor® 488-conjugated donkey anti-rabbit antibody (1:200, A21206, Invitrogen, Carlsbad, CA). Liver tissue sections were then mounted in an anti-fading medium.

Detection of serum aminotransferase activity

Whole blood samples were collected after bulbus oculi in mice with acute hepatic failure were exposed to light ether anesthesia. Serum was separated by centrifugation and stored at -30℃. Alanine aminotransferase activity was detected with an automatic biochemistry analyzer (Sinnowa D336, SINNOWA Medical Science & Technology Co., Ltd, Nanjing, China). The density of serum alanine aminotransferase (ALT) activity in experimental and control groups was compared. Serum ALT levels in experimental and control groups were measured in the following weeks.

Statistical analysis

Liver tissue from each mouse was cut into five sections. Each frozen section was examined under a microscope at a magnification × 200 under 10 microscopic fields. The total number of fluorescence-labeled cells in each section was calculated. At the same time, the serum aminotransferase activity was determined with an automatic biochemistry analyzer. Results were expressed as mean ± SD. Statistically significant differences between groups were compared with the *t* test. Paired *t* test was used to compare the PKH26 fluorescence intensity values and albumin expression. $P < 0.05$ was considered statistically significant. All data were processed using statistical software SPSS 10.0.

RESULTS

Extensive vacuolar degeneration and edema of hepatocytes were observed in mice of the experimental group, implying that an animal acute liver failure model can be successfully established.

Detection of PKH26-labeled cells

PKH26-labeled cells were detected in liver tissue sections following injection of SDF-1 *via* the tail vein. The total number of positive PKH26-labeled cells was 108 ± 8/high power field in the experimental group and 65 ± 8/high power field in the control group, respectively ($P < 0.05$, Figure 1B and Figure 2).

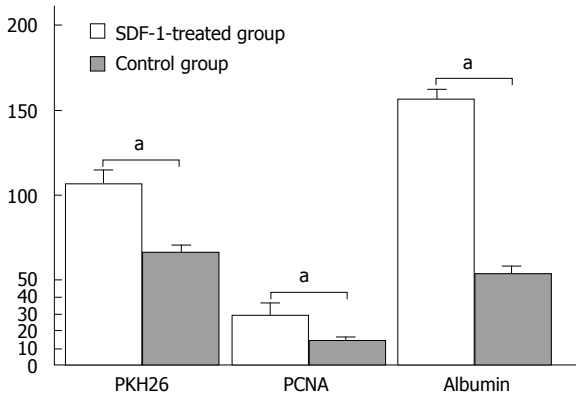

Figure 2 PKH26-labeled cells detected in experimental and control groups. Data are expressed as mean ± SD. $^aP < 0.05$ *vs* experimental group.

Detection of PCNA in the two groups

The expression level of PCNA was higher in the experimental group than in the control group. The total number of positive cells was 29 ± 7/high power field in the experimental group and 13 ± 2/high power field in the control group, respectively ($P < 0.05$, Figure 1C and Figure 2).

Detection of albumin in the two groups

The albumin expression level was also higher in the experimental group than in the control group. The percentage area of histologic field was calculated as previously described[7] and compared with albumin cells. The manual count set-up consisting of albumin image printouts and the transparent grid overlay used for point count are shown in Figure 1D. The total number of bone marrow stem cells at crossing points was 156 ± 5/high power field in the experimental group and 53 ± 5/high power field in the control group, respectively ($P < 0.05$, Figure 1D and Figure 2).

Green fluorescence was observed in FITC-labeled albumin antibodies at the 494 nm excitation light both in the experimental group and in the control group. Albumin in hepatocytes was detected with indirect labeling antibodies and expressed widely with green fluorescence. After the red and green fluorescence were focused, yellow fluorescence emerged at a suitable position, confirming that the yellow cells come from PKH26-labeled positive BMMC *in vitro* and show albumin (Figure 3).

It is well known that albumin is associated with the maturity of hepatocytes[8]. The obvious albumin expression in the experimental group implied that BMMC could differentiate into hepatocytes.

Detection of serum aminotransferase activity in the two groups

A significant difference in serum aminotransferase activity was observed between the two groups at different time points (120 ± 40 *vs* 118.50 ± 1.75, $P > 0.05$; 80.60 ± 6.50 *vs* 101.08 ± 5.67, $P < 0.05$; 50.74 ± 5.38 *vs* 80.47 ± 4.62, $P < 0.05$; 30.54 ± 2.70 *vs* 60.72 ± 4.37, $P < 0.05$; 30.77 ± 5.36 *vs* 40.47 ± 6.50, $P < 0.05$). At the same time, the serum AST levels in the experimental and

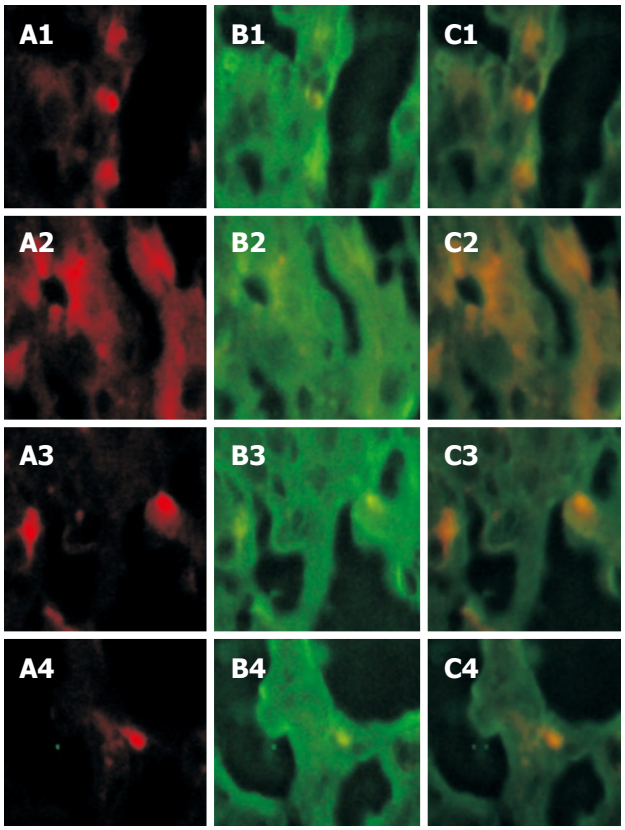

Figure 3 Confocal microscopy shows red fluorescence of cell location and green fluorescence of albumin. The red fluorescence cells could be found in liver tissue of recipient mice, suggesting that PHK-26 positive cells can emerge out of the red fluorescence (A1-A4). The albumin expressed in hepatocytes showed green fluorescence (B1-B4). After the red and green fluorescence cells were located, yellow cells were found in a suitable location (C1-C4).

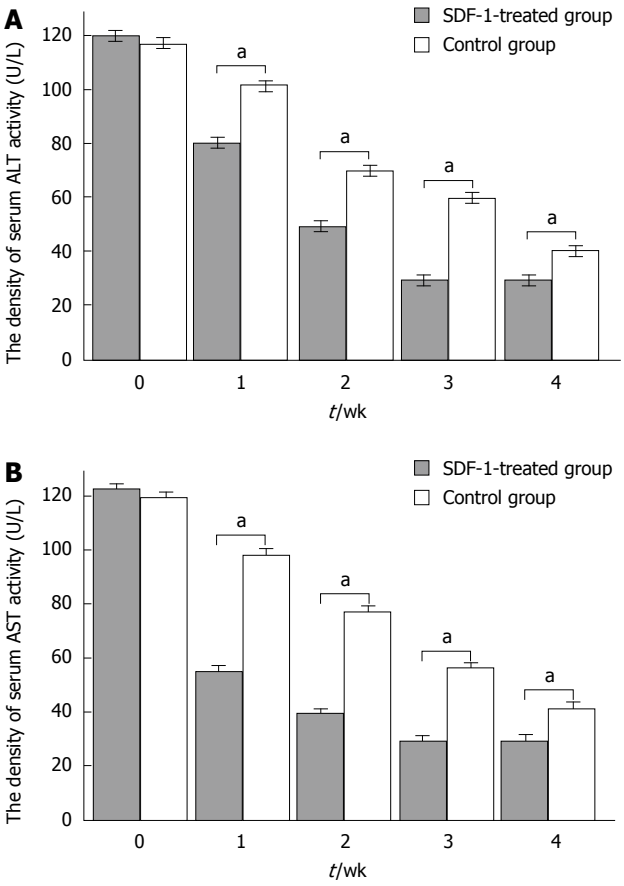

Figure 4 Detection of serum ALT (A) and AST (B) activity in the two groups. The serum ALT and AST levels were measured with an automatic biochemistry analyzer in experimental and control groups. There was a significant difference between the two groups. At the same time, the serum AST level was measured. $^{a}P < 0.05$ *vs* experimental group.

control groups were measured at different time points (122.55 ± 1.46 *vs* 120.70 ± 4.22, $P > 0.05$; 54.26 ± 6.50 *vs* 98.70 ± 8.20, $P < 0.05$; 39.47 ± 5.39 *vs* 78.34 ± 4.50, $P < 0.05$; 28.94 ± 2.70 *vs* 56.44 ± 4.28, $P < 0.05$; 30.77 ± 5.45 *vs* 42.50 ± 6.28, $P < 0.05$) (Figure 4A and B).

DISCUSSION

In regards to the genetic identity of inbred animals, use of BALB/c mice in the study allowed us to avoid immunologic rejection, thus the transplantation procedure represents an auto graft. Use of PKH26 fluorescent labeling permitted us to observe the homing of BMMC in the recipient group and to quantify the phenomenon by counting the fluorescencence-labeled cells as previously described[9]. By expressing albumin, the experimental data suggest that these cells can differentiate into hepatic cells. The expression of proliferating cell nuclear antigen suggested that the cells subsequently underwent cell division. PCNA is a nuclear antigen related with the cell life cycle, and is synthesized in cell nuclei. The PCNA is expressed in the G1 and S phases, and functions essentially as replicative DNA polymerases in eukaryotic cells[10]. The quantity of PCNA is low in resting cells but is substantially increased in multiplying and transformed cells. In this study, the expression levels of albumin and proliferating cell nuclear antigen were higher in the experimental than in the control group. Albumin was widely expressed in liver tissue. After red fluorescence was cofocused with green fluorescence, yellow fluorescence emerged at a suitable position, confirming that the yellow cells coming from PKH26-labeled positive BMMC *in vitro* can show albumin. Serum aminotransferase activity was detected. The density of serum ALT and AST was changed obviously after transplantation of BMMC, confirming that the liver function can be ameliorated by transplanted BMMC and the effect is more significant in the experimental group than in the control group, indicating that SDF-1 can promote BMMC homing to injured livers of mice.

BMMC mainly consist of HSC, BMSC and endodermis progenitor cells. Density gradient centrifugation was performed to remove adipocytes, erythrocytes and apocyte. BMMC were collected to enrich rudimentary bone marrow stem cells. SDF gene can code two proteins, namely SDF-1α and SDF-1β. The SDF-1α gene is expressed in bone marrow stromal cells. The homology of human SDF-1 and mouse SDF-1 can reach 99%[11]. In the study, the SDF-1 receptor gene (LESTER, CXCR4) carrying 7 membrane spanning domains, was

successfully cloned when the SDF gene, a kind of G protein linkage receptors, was cloned. CXCR4 is widely expressed in leucocytes, CD34+ HSC and CD34+ progenitor cells[12]. Initially, CXCR4 is regarded as a unique SDF-1 receptor[13] and the specific combination of SDF-1 and CXCR4, is named as SDF-1/CXCR4 biology axis. Another kind of SDF-1 receptors (CXCR7), known as an orphan receptor (RDC), has been recently found and is[14], mainly expressed in tumor cell line, activated endotheliocytes and fetal liver cells. CXCR7 is detected but not expressed in normal cells[15].

Aiuti *et al*[16] showed that SDF-1 is a chemotactic factor of CD34+ HSC, and CD34+ cells including endothelium progenitor cells, which can migrate and home along the concentration gradient of SDF-1. It has been shown that SDF-1 can play an important role in promoting migration of cells including endothelium progenitor cells from bone marrow to target tissue[17]. Bhakta *et al*[18] reported that marrow stromal cells can also express CXCR4 and SDF-1 is a chemotactic factor for the homing of marrow stromal cells *in vivo* and *in vitro*, suggesting that stem cells expressing CXCR4 can migrate and home along the concentration gradient. Moreover, the concentration gradient between inner and outer bone marrow can decrease the inner concentration gradient[19,20] or increase the outer concentration gradient[21] of bone marrow, thus promoting mobilization of bone marrow stem cells.

In the present study, the number of PKH26-labeled cells was obviously higher in the experimental group than in the control group and the expression level of albumin and PCNA was markedly higher in the experimental group than in the control group, demonstrating that SDF-1 can promote bone marrow stem cell migration into the liver and SDF-1 mobilized BMMC can be used to promote liver regeneration after liver injury. Moreover, use of SDF-1 can avoid immune suppression so that liver injury can be repaired. BMMC can be easily harvested and applied in clinical practice.

Unfortunately, the mechanism by which SDF-1 becomes chemotactic to bone marrow stem cells is unclear. It has been shown that SDF-1 can ignite multiple signal pathways in cells and is regulated by different regulatory factors[22-24]. When SDF-1 binds to CXCR4, certain second messengers, such as NO and IP_3, lead to a series of related kinase phosphorylation[23] and the production of actin, and rapid or transient polymerization of biological effects by changing the hereditary information of stem cells[22], all of which may be due to the stem cell migration induced by SDF-1.Protein kinase B, ecto-signal regulatory protein-2 and JAK2 also participate in the signal conduction pathways[24].

In addition, SDF-1 can act as a chemoattractant to promote migration of stem cells[25,26] and strengthen their locomotory capacity[27]. When stem cells are migrated to the target tissue, SDF-1 facilitates their adhesion to fibrinogen, fibronectin, interstitium and endotheliocytes[28]. Then the cells, adhered to blood vessel endothelium, permeate vessel walls to ingress target tissue. With the help of SDF-1, more secreted MMP-9, NO and VEGF promote the mobilization of stem cells[23,29,30]. It has been reported that SDF-1 may participate in tumor development[31,32].

In conclusion, with the rapid progress in the field of stem cells, various kinds of stem cells are widely used to treat different organ diseases. SDF-1 can play an important role in bone marrow stem cells expressing CXCR4, thus promoting migration of BMMC into liver tissue. However, the precise mechanism by which SDF-1 mobilizes stem cells is unclear. Further study is needed to determine the dose, administration route and safety of SDF-1.

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COMMENTS

Background

Stem cells have the potential ability of multi-directional differentiation and self-renewal. Under proper induction circumstances, stem cells can differentiate into different functioning cells. Differentiation between the groups is ongoing. The new treatment strategy for acute and chronic hepatitis is of potential importance.

Research frontiers

Stromal cell derived factor-1 (SDF-1), a kind of micro-molecular proteins, possesses a variety of biologic activities. It has been shown that SDF-1 can promote bone marrow stem cell directional differentiation into heart and nerve tissues. The results of this study demonstrated that SDF-1 could promote the homing of bone marrow mononuclear cells (BMMC) to the liver of mice with acute liver failure.

Innovations and breakthroughs

Recent reports have highlighted that the mesenchymal stem cells in human second-trimester bone marrow, liver, lung, and spleen exhibit a similar immunophenotype, but a heterogeneous multilineage differentiation potential. We hypothesize that SDF-1 can also mobilize the homing of bone marrow mononuclear cells in mice with acute liver failure. This is the first study to report that SDF-1 can promote the homing of BMMC to the liver in mice with acute liver failure.

Applications

This study showed how SDF-1 promotes the homing of BMMC to the liver, thus providing a future strategy for therapeutic intervention in the treatment of acute liver failure.

Terminology

CXCL12 is a recombinant murine SDF-1-α. SDF-1-α and β are small cytokines belonging to members of the intercrine family, can activate leukocytes, and are often induced by proinflammatory stimuli such as lipopolysaccharide, TNF, or IL-1.The intercrines are characterized by the presence of four conserved cysteines which form two disulfide bonds, and can be classified into two subfamilies. In the CXC subfamily including β and α chemokines, cysteine residues are adjacent to each other and separated by an intervening amino acid, respectively. SDF-1 proteins belong to the latter group.

Peer review

In this manuscript, SDF-1 was found to facilitate migration of infused bone marrow cells to liver. The total number of PKH26-labled bone marrow cells in the liver was higher in the experimental group than in the control group. Moreover, the number of albumin-producing cells and proliferating cells was higher in the experimental group than in the control group. Although the data are preliminary, they are important and encouraging.

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CASE REPORT

Bone and brain metastases from ampullary adenocarcinoma

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Abstract

Ampullary carcinoma is the second most common cancer of the peri-ampullary area after pancreatic carcinoma and metastasizes mostly intra-abdominally and to the liver. Extra-abdominal metastases are less frequent. In this report we describe the case of a patient with resected adenocarcinoma of the ampulla of Vater who developed skeletal metastases in the lower extremity and brain metastases. We briefly discuss aspects of this comparatively rare gastrointestinal malignancy.

Key words: Adenocarcinoma; Ampulla of Vater; Peri-ampullary; Metastases; Prognosis

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INTRODUCTION

The ampulla of Vater includes a common channel derived from the conjunction of the common bile duct and the pancreatic duct, the intra-duodenal parts of these two ducts and the duodenal mucosa surrounding the opening[1]. Peri-ampullary carcinomas arise from the ampulla of Vater, distal bile duct, pancreas or duodenal epithelium and are managed with surgical excision when resectable. Regional and other abdominal lymph nodes and the liver are the primary metastatic sites. Other sites of metastasis are less common but can be encountered especially in long term survivors.

We present the case of a patient with ampullary carcinoma and local recurrence developing both biopsy-proven bone metastases in unusual locations and brain metastases.

CASE REPORT

A 62-year-old woman presented to the orthopedic clinic complaining of painful localized edema involving the right ankle and shin. Two years earlier, she had undergone surgery for a stage ⅡA (pT3N0M0) low grade ampullary adenocarcinoma. She had developed mesenteric and retroperitoneal lymph node metastases 4 mo postoperatively. At that time the patient had refused chemotherapy since she was asymptomatic.

Diagnostic work-up of leg symptoms included an X-ray of the right foot and tibia, which revealed an ill-defined radio-opaque lesion of the right cuneiform bone and a radiolucent lesion of the tibia (Figure 1). An MRI scan of the right lower extremity disclosed multiple confluent bone erosions at the diaphysis of the tibia, with significant extention both proximally and distally. A technetium bone scan showed increased uptake in the right foot and tibia but no other skeletal lesions (Figure 2). In view of the unusual location of the metastasis, a bone biopsy was performed and revealed metastatic adenocarcinoma similar to the primary ampullary tumor. External beam irradiation was given locally (2000 cGy total in five fractions) for pain palliation.

A follow-up staging CT of the chest, abdomen and pelvis revealed two liver lesions, disseminated bilateral metastatic pulmonary nodules as well as borderline enlarged lymph nodes across the aortic arch and trachea bifurcation. At this time the patient consented to palliative chemotherapy and gemcitabine 1000 mg/m^2 iv weekly was administered.

Six months later the patient developed changes in mood, and behavior. A brain CT scan demonstrated

Figure 1 X-ray. A: The right foot showing the radio-opaque metastatic lesion in the cuneiform bone; B: The right tibia showing a radiolucent metastatic lesion.

Figure 2 A whole body bone scan showing increased uptake in the right foot and tibia.

ring enhancing lesions affecting the right frontal and left temporal lobes as well as the left cerebellar hemisphere. Palliative whole brain radiation followed by palliative targeted therapy with erlotinib 150 mg/d *po* was administered. The patient died with progressive disease 7 mo later and about 3 years after the diagnosis of her disease.

DISCUSSION

Carcinoma of the ampulla of Vater is a rare tumor accounting for approximately 0.2% of all gastrointestinal malignancies with an estimated incidence of less than 6 cases per million people per year. However, it is the second most common peri-ampullary neoplasm following pancreatic carcinoma in incidence. The peak incidence is in the 7th and 8th decades of life. There is a slight male to female preponderance[2].

The adenoma to adenocarcinoma carcinogenesis sequence model seems to be applicable to these tumors, given that the incidence of adenoma surrounding carcinoma of the papilla of Vater ranges from 82% to 91%[3]. Intestinal and pancreaticobiliary types represent the most common histological subtypes, indicating that peri-ampullary carcinomas emanate from the corresponding epithelia covering the distal parts of pancreatic and common bile duct and peri-ampullary duodenum, respectively[2]. A more favorable prognosis of the intestinal sub-type compared with the pancreatico-biliary type has been observed in some series[1,4,5]. Other histologic types less commonly seen in an ampullary location include mucinous, signet-ring cell, neuroendocrine and undifferentiated carcinomas[2,6-9].

Compared to other upper GI tract sites, as well as its periampullary counterparts, ampullary cancer has a favorable prognosis possibly due to the relatively early presentation with jaundice, rendering it resectable in 80%-90% of cases[10,11]. Moreover molecular lesions of ampullary carcinoma are different from other peri-ampullary cancers, a fact that may also contribute to the different prognosis[12]. For example k-ras, which is mutated in 90% of pancreatic cancers, is mutated in only half of the ampullary carcinomas. Overall the 5-year survival rate ranges from 33% to 67.7% in resected cases[10,12-14]. Tumor size and grade, lymph node status and number of positive nodes, perineural infiltration and surgical margins are the most crucial predictors of survival in localized disease[15-18]. Vascular invasion, ulcerative tumor, pancreatic invasion, age and peri-operative blood transfusions are additional prognostic factors seen in some series[18-21]. Nodal involvement is the most robust predictor of survival and forms the basis for recommending adjuvant radiotherapy and chemotherapy to decrease the incidence of locoregional recurrence and distal metastases respectively. Expression of specific proteins by immunohistochemistry has also been linked to prognosis. Expression of transcription factor CDX2 (Caudal-type homeodomain transcription factor), a regulator of normal intestinal and colonic epithelial differentiation, has been linked to improved prognosis compared with ampullary tumors that do not express the transcription factor[22]. In contrast high expression of the transporter protein hENT1 (human Equilibrative nucleoside transporter 1) has been shown to correlate with a shorter survival compared with ampullary carcinomas that displayed low expression of the transporter[23].

Ampullary cancer usually metastasizes to regional nodes, liver, adjacent organs and lungs. In contrast skeletal and brain metastases are common with other primary tumor locations such as lung and breast. We present a patient with an ampullary tumor that developed both bone metastases in unusual locations and brain metastases during the course of her disease. Bone and brain metastases from ampullary carcinoma are not very commonly seen in practice and are rarely reported in the literature. In a series of 135 patients with ampullary cancer who had previously undergone pancreaticoduodenectomy, bone metastases were seen in 5% and brain metastases in less than 4%[13]. In a smaller series of 14 cases of carcinomas of the ampulla of Vater, two had brain metastases but they concerned patients with carcinomas with neuroendocrine differentiation[8], while bone metastases were reported in three cases of another series of 24 patients (13%)[24].

Other isolated cases of bone metastases and unusual metastatic locations, such as the ureter, ovaries, testes, bronchus, umbilicus and distal lymph nodes have also been reported[25-30]. The specific location of bone metastases from ampullary carcinoma are not in general

reported and, thus, it is unknown if the common sites seen in several malignancies such as the spine, pelvis and proximal lower extremities are also the most frequent sites of bone metastases in this malignancy.

Although no randomized trials have been performed to support adjuvant treatment, local radiation therapy concomitant with 5-fluoropyrimidine-based chemotherapy has been advocated for resected ampullary carcinoma with adverse prognostic features such as size greater than 2 cm, positive lymph nodes, positive surgical margins, poor differentiation and neurovascular invasion[31,32]. In one of these series with 12 patients using protracted infusion of 5-FU concomitantly with radiation treatment, a 2 years survival rate of 89% and a median survival time of 34 mo was reported[32]. In another series a statistically significant improvement in 3 years survival was observed with adjuvant chemoradiotherapy compared with no adjuvant treatment only in patients with high risk characteristics but not in the whole group[31]. In metastatic disease palliative chemotherapy is based on pancreatic cancer type regimens with fluoro-pyrimidines, gemcitabine and platinum derivatives. Resection of solitary liver metastases in an attempt at palliation and prolongation of survival has been done and should be considered in appropriately selected patients[33].

In conclusion, despite their infrequency, bone metastases in the extremities and brain metastases should be included in the differential diagnosis of patients with a history of ampullary carcinoma who present with symptoms referring to such locations in order to expedite the diagnosis and facilitate treatment.

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CASE REPORT

Ceftriaxone-induced toxic hepatitis

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Abstract

Toxic hepatitis or drug-induced liver injury encompasses a spectrum of clinical disease ranging from mild biochemical abnormalities to acute liver failure. The advantages of a long half-life, wide spectrum, high tissue penetration rate, and a good safety profile, make ceftriaxone, a third-generation cephalosporin, a frequent choice in the treatment of childhood infections. Previous studies have reported a few cases of high aspartate aminotransferase and alanine aminotransferase levels, along with three cases of hepatitis caused by ceftriaxone. Here, we report a case of drug-induced toxic hepatitis in a patient who was treated with ceftriaxone for acute tonsillitis.

Key words: Hepatitis; Toxic; Ceftriaxone; Children; Drug

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INTRODUCTION

Toxic hepatitis or drug-induced liver injury encompasses a spectrum of clinical disease ranging from mild biochemical abnormalities to acute liver failure. The majority of adverse liver reactions are idiosyncratic, and occur in most instances 5-90 d after the causative medication was last taken[1]. The advantages of a long half-life, wide spectrum, high tissue penetration rate, and a good safety profile, make ceftriaxone, a third-generation cephalosporin, a frequent choice in the treatment of childhood infections. It is excreted mainly by the kidneys, and 35%-45% of the drug is excreted through the bile without being metabolized[2]. Previous studies have reported a few cases of high aspartate aminotransferase (ALT) and alanine aminotransferase (AST) levels, along with three cases of hepatitis caused by ceftriaxone[2-4]. Here, we report a case of drug-induced toxic hepatitis in a patient who was treated with ceftriaxone for acute tonsillitis.

CASE REPORT

A 12-year-old boy was admitted with complaints of weakness and fatigue. His personal history revealed treatment with ceftriaxone 50 mg/kg per day, 6 d previously, for tonsillitis. The patient stated that the weakness had begun on the third day of ceftriaxone therapy. He had no chronic disease and had not had contact with anyone with hepatitis. He had not used any drugs, including analgesics and anti-inflammatory drugs, in the previous 6 mo. He had never undergone a blood transfusion or any previous operations. or tooth extraction in the previous year. He had no history of traveling. He was a student in the 6th grade of primary school. He stated that there was no change of color in his feces or urine. During this period, other medications, including herbal remedies and vitamins had not been used. The only drug he had used was ceftriaxone. His liver function tests were at normal levels in the past 2 mo. Physical examination revealed a weight of 43 kg (50 percentile), and a height of 152 cm (50-75 percentile). His general appearance was moderate and seemed weak, and his pharynx was slightly hyperemic. No additional sounds or murmurs were detected upon cardiac and pulmonary examinations. The liver and the spleen were

non-palpable. The neurological examination was normal, along with all the other systems.

Laboratory examination revealed: AST, 819 IU/L (normal range, 10-40 IU/L); ALT, 871 IU/L (normal range, 13-40 IU/L); γ glutamyl transferase (GGT), 285 U/L (normal range, 9-50 IU/L); alkaline phosphatase (ALP), 143 IU/L (normal range, 40-140 IU/L); total bilirubin, 4.2 mg/dL; and direct bilirubin, 2.8 mg/dL. Total protein, albumin, globulin, lactate dehydrogenase, amylase and fasting blood glucose levels were normal. The complete blood count revealed a normal number of leukocytes, erythrocytes and platelets. Peripheral blood smear revealed 60% neutrophils, 30% lymphocytes, 8% eosinophils, and 2% monocytes. The erythrocyte morphology was normal with no atypical cells. Urine analysis, prothrombin time and activated partial thromboplastin time were all in the normal range. Antistreptolysin O titer was 340 TU/mL. Serum iron, iron binding capacity, ferritin, ceruloplasmin, free T3, free T4 and thyroid stimulating hormone levels were also in the normal range. Hepatitis B surface antigen, anti-hepatitis B core IgM, anti-hepatitis C virus, anti-hepatitis A virus IgM, anti-hepatitis E virus, cytomegalovirus IgM, and Epstein-Barr virus viral capsid antigen values were negative, whereas anti-HbsAg was positive. Values for IgG, IgM and IgA levels were normal but the value for IgE was 523 IU (normal range 0-100). Anti-nuclear antibody (ANA) and anti-mitochondrial antibody (AMA) were negative. Blood, urine and throat cultures were negative.

Ultrasonography showed minimal enlarged liver size but with normal parenchyma, and gallbladder was normal. The clinical appearance of the patient did not show any signs of cholelithiasis. Evaluating personal history, physical examination and the laboratory findings together, we made a diagnosis of ceftriaxone-induced hepatitis. Liver biopsy was planned, but the patient refused. Ceftriaxone administration was ceased immediately. Pulse methylprednisolone administration was begun at 40 mg/kg in the first 3 d, to be followed by 30 mg/kg for 4 d. A proton pump inhibitor was added to the drug regimen. His vital functions were normal. A steroid-induced hyperglycemic attack with the highest value of 240 mg/dL for 2 d was the only adverse effect of the treatment. He was advised to rest for 2 wk without taking any drugs and to have a light diet. At week 4, the biochemical data revealed: ALT, 95 IU/L (13-40); and GGT, 164 IU/L (9-50). Total bilirubin, direct bilirubin, total protein, albumin and other parameters were normal. Control blood biochemistry at week 10 showed GGT of 85 IU/L (9-50), along with normal values of ALT, AST and other parameters.

DISCUSSION

All the factors suspected to be responsible for hepatitis and disturbed liver function tests, such as viral agents, autoimmune disease, cholelithiasis, storage diseases such as Wilson's disease, hemochromatosis, and endocrine diseases such as hypo- and hyperthyroidism were excluded when we obtained normal values in viral serological tests of ANA, AMA, ferritin, serum iron binding capacity, ceruloplasmin, free T3, free T4, and TSH.

The absence of a specific cause for the elevated liver function tests, including AST, ALT, ALP, GGT, and total and direct bilirubin, such as blood transfusion, recent tooth extraction, surgery, direct contact with a patient with hepatitis, history of traveling or use of any drugs other than ceftriaxone, other medications, including herbal remedies and vitamins, led us to consider ceftriaxone as the responsible agent. The direct correlation with ceftriaxone use could be verified by measurement of drug levels in the serum or by liver biopsy, or the re-use of the drug, in which case elevated transaminase levels would support our diagnosis of ceftriaxone-induced hepatitis. Measurements of the antibody for liver-kidney microsome (anti-LKM) and cytochrome P450 may be useful for demonstrating drug-induced hepatotoxicity[4]. Re-use of the drug in our patient was out of the question. He also refused a liver biopsy. In our case, serum measurement of anti-LKM was positive but for technical reasons, serum ceftriaxone levels could not be performed. A prominent increase in the levels of GGT suggest a toxic cause[3].

Development of hepatitis and elevated liver enzymes caused by antibiotics have been reported in the literature[2-9]. Cephalosporin-induced hepatoxicity is rarely observed. Common adverse effects are gallstones (cholelithiasis) or bile lumps. Despite the fact that only a few cases of elevated liver enzymes caused by ceftriaxone have been reported[2-4], only three cases of hepatitis have been reported in the literature[2-4]. In one of the cases, ceftriaxone was used to treat Lyme's disease, which resulted in serious side effects and elevated liver enzymes, consequently leading to cessation of the drug[4]. Similar to our case, an interesting normalization in the level of all the enzymes, except GGT, has been reported at 10 wk after drug discontinuation[4]. Unlike other cases, our patient showed no signs of severe hemolysis. This probably accounted for early recovery of the patient, along with the absence of any life-threatening complications. This was certainly fortuitous for the patient.

In cases of drug-induced hepatitis, the clinical picture of the hepatitis may represent a direct toxic effect, an idiosyncrasy, or a cholestatic reaction[1,5]. In our case, the eosinophilia in the blood smear and the elevated IgE levels in the serum suggested that hypersensitivity was responsible for the ceftriaxone-induced hepatitis.

In conclusion, the effect of ceftriaxone along with other hepatotoxic drugs should be considered in any case of elevated liver enzymes and hepatitis.

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CASE REPORT

An unusual cause of ileal perforation: Report of a case and literature review

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Abstract

An ileal perforation resulting from a migrated biliary stent is a rare complication of endoscopic stent placement for benign or malignant biliary tract disease. We describe the case of a 59-year-old woman with a history of abdominal surgery in which a migrated biliary stent resulted in an ileal perforation. Patients with comorbid abdominal pathologies, including colonic diverticuli, parastomal hernia, or abdominal hernia, may be at increased risk of perforation from migrated stents.

Key words: Adhesion; Biliary stent; Ileal perforation; Migration; Choledocholithiasis

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INTRODUCTION

The endoscopic placement of biliary stents for benign and malignant biliary disease has been performed for well over a decade and was first described in 1980 by Soehendra and Reynders-Frederix[1,2]. In approximately 5%-10% of cases, the procedure itself involves potential adverse side effects, such as pancreatitis, hemorrhage, perforation, and cholangitis. Long-term complications of biliary stents, such as migration and perforation, are unusual[2]. Patients with comorbid abdominal pathologies, including colonic diverticuli, parastomal hernia, or abdominal hernia, may be at increased risk of perforation from migrated stents. The available biliary endoprostheses can be classified by material into two categories: plastic and metallic stents. Plastic stents are less expensive and easier to remove or change, but have a higher risk of clogging and dislocation[3,4].

Generally, to prevent migration or clogging in patients with a plastic stent, the stent must be changed or removed in 3-6 mo[5]. Moreover, to avoid stent migration, the biliary stent should be placed across the sphincter of Oddi[6].

CASE REPORT

A 59-year-old woman was admitted to the hospital with increasing abdominal pain. Her abdomen was tense and painful, with diffuse abdominal rebound tenderness and hyperactive bowel sounds. An upright abdominal radiograph showed minimally dilated small bowel loops and no free intraperitoneal gas. Laboratory examination gave the following results: hematocrit 30%, white blood cell count 14 500/cm^3, platelets 268 000/cm^3, sodium 138 mEq/L, potassium 4.6 mEq/L, blood urea nitrogen 32 mg/dL, creatinine 1.6 mg/dL, glucose 110 mg/dL, amylase 78 IU/L, total bilirubin 2.5 mg/dL, aspartate aminotransferase 87 IU/L, alanine aminotransferase 79 IU/L, alkaline phosphatase 550 IU/L, and γ glutamyl transferase 66 U/L.

An abdominal ultrasound showed features consistent with acute cholecystitis and a dilated common duct with a diameter of 14 mm. Computed tomography (CT) was performed because of the extensive pain. Dislocation and migration of a biliary stent to the distal ileal segment was detected. CT was suspicious for an ileal perforation and the wall of the ileum was markedly thickened, with

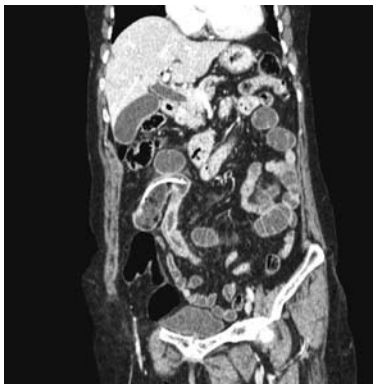

Figure 1 The coronal MIP abdominal CT views show the stent in the ileum lumen indenting the ileum wall; proximal to this level, the intestinal segments are dilated.

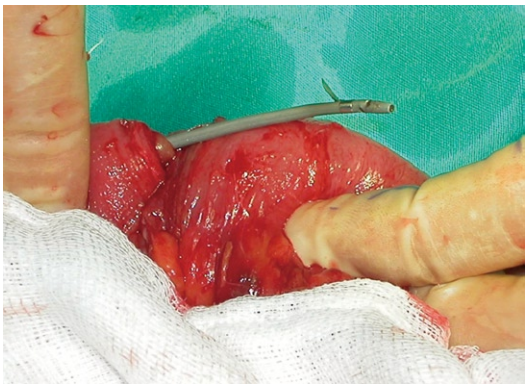

Figure 2 Intraoperative photograph shows the antimesenteric surface of the terminal ileum perforated by the biliary stent.

choledocholithiasis and dilated common duct with a diameter of 14 mm (Figure 1). The patient's history included endoscopic retrograde cholangiopancreatography (ERCP) 21 d before admission for an impacted stone in the common bile duct. A sphincterotomy was performed and a plastic 7-cm 11 Fr stent was placed. The patient had also undergone a laparotomy for appendicitis 20 years earlier, and three repairs of incisional hernias, 3, 4 and 6 years previously.

At laparotomy, we found multiple adhesions, dilated proximal jejunal segments, and a large amount of small intestine conglomerated in the right lower quadrant. The biliary catheter had perforated the antimesenteric surface of the distal ileum, which resulted in a protruding plastic biliary stent (Figure 2), however, the contamination was moderate because of the dense intra-abdominal adhesions. Two liters of turbid fluid were aspirated. The biliary stent was removed; the small bowel, including the perforated region, was partially resected. The patient was discharged on the seventh postoperative day, with no complications.

DISCUSSION

Occlusion is the most common problem associated with endoscopically placed stents (54%); this can be caused by tumor overgrowth or clogging by "biliary sludge," with resulting cholangitis or recurrent obstructive jaundice[1]. Dislocation and migration of biliary stents proximally and distally in the gut is less common, occurring in less than 10% of cases, and generally causes no major problems, with the stent usually passing in the feces or remaining in the bowel with no overt symptoms. Migration is much more common with plastic stents than with metallic stents[1,7].

In a series of 322 endoscopically placed biliary stents, Johanson *et al*[8] reported proximal and distal stent migration in 4.9% and 5.9% of the patients, respectively. Intestinal perforation is an exceedingly rare complication after placement of a biliary stent. Intestinal perforation can occur during the initial insertion or endoscopic or percutaneous manipulation, or as a late consequence of biliary stent placement. In the recent literature, most (92%) cases of intestinal perforation were in the duodenum after endoscopic or percutaneous placement of a biliary stent[4,5,9,10]. Various mechanisms have been postulated for duodenal perforation by biliary stents. Firstly, the stent may be placed incorrectly at the time of the procedure, and the mechanical force exerted by the tip of the plastic stent against the duodenal mucosa can lead to necrosis of the wall over time. Secondly, inflexibility or a stent of an incorrect length may lead to pressure necrosis[1,9].

The insertion of several smaller-diameter stents (10 Fr) rather than a single large stent (11.5 or 12 Fr) may prevent proximal stent migration. The insertion of longer rather than shorter (< 7 cm) stents may also prevent such migration. Distal perforations are less common than duodenal perforations. Reported cases involve perforation due to biliary stent migration; this is more unusual in the distal ileum and generally the cause is other diseases, such as colonic diverticuli, parastomal hernia, incarcerated hernia, and colovesicular fistulas[1,8,10]. In our case, many adhesions resulting from the previous surgery were observed and the ileal segments were conglomerated in the right lower quadrant. These adhesions reduced the mobility of the ileum and the stent was caught in a distal ileal segment. Consequently, the sharp edge of the stent caused irritation and perforation on the antimesenteric side of the distal ileum. In the coronal maximum intensity projection abdominal CT reconstructions, the localization of the stent was obvious (Figure 1).

A review of the literature published to January 2007 revealed 11 cases of colonic perforation due to biliary stent migration, with the majority being straight plastic endoprostheses[3]. Five cases of small intestinal perforations due to a migrated biliary stent were reported, which involved an incisional hernia, parastomal hernia, appendiceal perforation, and two other diseases[1,11-14]. To our knowledge, this is the sixth case of small intestinal perforation due to a migrated biliary stent reported in PubMed. In our case, the clinical manifestations appeared 21 d after placing the stent *via* ERCP.

In conclusion, patients with risk factors such as diverticulosis, incisional and incarcerated hernias, and intra-abdominal adhesions require more attention.

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CASE REPORT

Cardiac metastasis from colorectal cancer: A case report

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Abstract

The heart is an unusual site of metastasis from any malignancy. We report a case of cardiac metastasis from colorectal cancer. A 70-year-old woman was referred with a presumptive diagnosis of sigmoid colon cancer with cardiac myxoma. Two-dimensional echocardiography showed a 4 cm × 4.5 cm mobile mass on the lateral right atrial wall, and computed tomography revealed a low attenuated lobulating mass in the right atrium. The patient underwent anterior resection for sigmoid colon cancer (T4N2). Thereafter, she experienced progressive shortness of breath. Therefore, a cardiac operation was performed 2 wk after the colorectal operation. Histological examination revealed adenocarcinoma, which was identical to the primary lesion. Although two-dimensional echocardiography has become the diagnostic test of choice for detecting cardiac tumors, in patients with colorectal cancer showing a cardiac mass, further diagnostic evaluation such as a magnetic resonance imaging might be necessary.

Key words: Heart; Cardiac metastasis; Colorectal cancer; Atrial myxoma; Magnetic resonance imaging

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INTRODUCTION

Metastases from colorectal cancer can occur either by lymphatic or hematogenous spreading, and the sites most commonly involved are the liver and lung. Unusual metastases from colorectal cancer into organs including the spleen, thyroid gland, spermatic cord, and skeletal muscle have been reported[1-4]. Although metastases to these sites might occur as a feature of end stage-disease, metastasis to the heart from colorectal cancer is extremely rare. To our knowledge, only nine cases of cardiac metastasis from colorectal cancer have been previously described[5-9,10-13]. Here, we report a case of cardiac metastasis from colorectal cancer that presented preoperatively as a benign atrial myxoma.

CASE REPORT

A 70-year-old woman, presenting with bloody stools, was admitted to our hospital. She experienced shortness of breath and had lost 4 kg in weight in the past 3 mo. A colonoscopy revealed an encircling mass in the sigmoid colon, nearly obstructing the lumen of the colon. A pediatric colonoscope could not be passed beyond this point. The biopsy gave evidence of an adenocarcinoma of the sigmoid colon. Laboratory studies, on admission, were within the normal limits, except for carcinoembryonic antigen, which was 9.2 ng/mL. A routine transthoracic echocardiography showed the presence of a right atrial (RA) enlargement and mobile, round, spherical, and inhomogenous mass (3.72 cm × 4.15 cm) adjacent to the lateral RA wall (Figure 1). This pedunculated mass had a broad stalk (1.88 cm), and projected through the tricuspid valve into the right ventricular (RV) cavity, obstructing the RV inflow during the diastolic phase. A chest X-ray showed cardiomegaly and a prominent aorta. Computed tomography revealed a low attenuated lobulating mass in the right atrium (Figure 2). Preoperative diagnosis

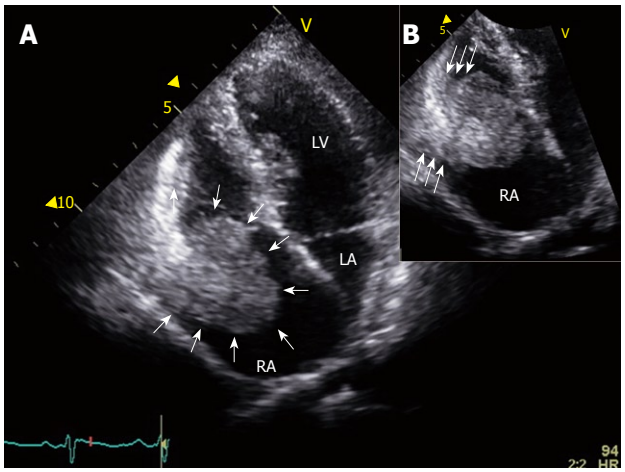

Figure 1 Transthoracic echocardiography. A: A mobile, round, spherical, and echogenic mass (white arrows) is seen adjacent to the lateral right atrial wall on an apical four chamber view of the transthoracic echocardiography; B: A magnified image of the right atrial mass shows a pedunculated character with a broad stalk (tris-arrows). RA: Right atrium; LA: Left atrium; LV: Left ventricle.

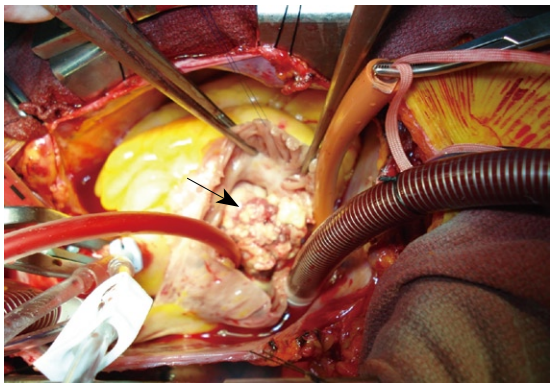

Figure 3 Operative finding. On opening the right atrium, a large multiple lobulating mass (arrow) with a rough surface was located on the antero-inferior side of the right atrial free wall. The mass was near the atrioventricular groove, with invasion into the right atrium.

was synchronous sigmoid colon cancer and right atrial myxoma. We planned to perform cardiac surgery 4 wk after colorectal surgery. An anterior resection with a colorectal anastomosis was performed. Histological investigation of the excised portion confirmed the existence of a moderately differentiated adenocarcinoma invading the serosa with lymphovascular invasion and perineural invasion. The staging of the lesion was T4N2. Although the patient complained of mild shortness breath, the postoperative course was uneventful. However, 2 wk postoperatively, the shortness of breath had worsened. Thus, the patient was taken immediately into cardiac surgery. After a sternotomy, the pericardium was opened, and a cardiopulmonary bypass was installed to open the right atrium. The mass was present in the right atrial wall near the atrioventricular groove, and was growing toward the tricuspid valve. After removal of the mass, the right atrial wall was reconstructed with bovine pericardium (Figure 3). The atrial mass showed a nodular necrotic appearance grossly measuring

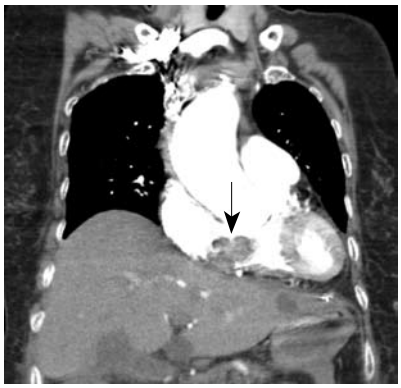

Figure 2 Computed tomography scan revealed that the mass (arrow) was located in the right atrium, obstructing the tricuspid valve opening.

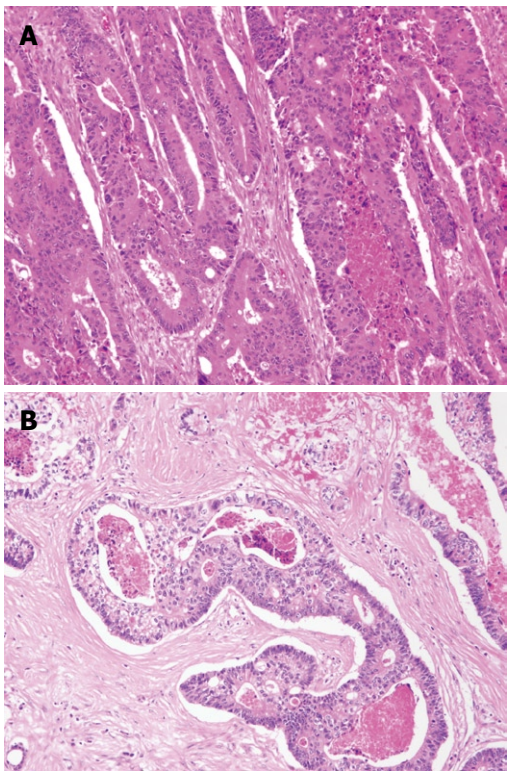

Figure 4 Microscopic findings. A: Tall malignant columnar cells line large irregular glands; some forming a cribriform architecture containing intraluminal necrotic debris in primary colon cancer (HE, × 100); B: The cardiac mass shows similar histological findings to Figure 4A.

5.5 cm × 5 cm × 3 cm; histological examination revealed adenocarcinoma that was identical to the primary lesion (Figure 4). A further operation was performed because of postoperative cardiac bleeding; however, the patient died of recurrent cardiac bleeding on the 3rd postoperative day.

DISCUSSION

The regional lymph nodes, liver, and lung are the most common sites of metastasis from colorectal cancer. Infrequent sites of metastases, including the spleen, thyroid gland, spermatic cord, and skeletal muscle

Table 1 Clinical features of cardiac metastasis from colorectal cancer reported in English literature

Author (yr)	Age/sex	Location of primary tumor	Primary tumor stage	Diagnosis of cardiac metastasis made by/at	Location of heart	Metastatic tumor size (cm)	Operation of metastatic mass	Outcome
Henuzet (1982)[13]	60/M	Rectum	NA	Echocardiography	Right ventricle	2	Resection	Dead
Massachusetts General Hospital (1992)[12]	75/M	Colon	NA	Autopsy	Right ventricle	7.5 × 4.5 × 4	No	Dead
Parravicini (1993)[8]	47/M	Rectum	NA	Surgery	Right ventricle	10 × 4 × 3.5	Resection	Dead
Testempassi (1994)[7]	71/F	Colon	Stage Ⅲ	Magnetic resonance imaging	Right ventricle	NA	NA	NA
Lord (1999)[6]	71/M	Rectum	Dukes′ C	Biopsy	Right ventricle	NA	No	Dead
Koizumi[11] (2003)	65/M	Rectum	Dukes′ C	Surgery	Right atrium	6 × 5	Resection	Dead
Present case (2009)	70/F	Colon	T4N2	Surgery	Right atrium	5.5 × 5 × 3	Resection	Dead

NA: No available information; M: Male; F: Female.

have been reported[1-4]. Although metastases of these unusual sites from colorectal cancer might occur with the status of widespread disease, cardiac metastasis from colorectal cancer is extremely rare[5-9,10-13]. The incidence of cardiac metastasis in patients with malignancy might be underestimated because cardiac metastatic lesions are clinically silent in most cases[13,14]. Therefore, the incidence of cardiac metastasis might best be determined by reviewing autopsy studies. The incidence of cardiac metastasis from any malignancy has been reported as 10%-18% in autopsy studies[15-17]. Malignant melanoma has been reported as the most common disease[16-19]. Leukemia and lymphoma also showed a high incidence of cardiac metastasis in the past, but this incidence is decreasing because of improvements in chemotherapy[16,20]. In colorectal cancer, to our knowledge, only nine reports have been described in the literature (Table 1); however, the incidence of cardiac metastasis was 1.4%-7.2% in autopsy studies[12,16,17]. Thus, the true incidence of cardiac metastasis from colorectal cancer might be higher than the cases reported so far.

Some factors that might account for the infrequency of cardiac metastasis have been suggested: the strong kneading action of the myocardium, metabolic peculiarities of striated muscle, the rapid flow of blood through the heart, and lymph flow normally moving away from the heart. The blood flow of the coronary system is the highest in the body, and the lymphatics of the heart is divided into a superficial and a deep group, joining and draining through two trunks into the tracheobronchial lymph nodes. Thus, metastasis must occur against the direction of flow[19]. Although the reasons for its rarity have not been well established, cardiac metastasis from malignancies might occur by direct extension, lymphatic spread, hematogenous spread, or a combination of two or three of the above[14,16,17,19]. Klatt *et al*[16] reported that the lung was the most common primary site, and sites closer to the heart contributed to the greatest number of cardiac metastases; they suggested that the lymphatic drainage from nearby sites represents the most likely means of spread. Mukai *et al*[17] suggested that the relative importance of these various routes would appear to depend on the type of primary tumor and on the completeness of the autopsy examination. In our case, although regional lymph node metastases were identified, we postulate that hematogenous spread might be the most likely route in colorectal cancer.

The incidence of colorectal cancer with cardiac metastasis is rare, therefore surgery as a treatment modality has not been investigated. Koizumi *et al*[11] reported that although surgery is rarely recommended for treating metastatic cardiac tumors, surgical treatment could be especially effective in occurrences of obstructive and solitary lesions to ensure relief from symptoms and elongation of life expectancy. In our case, the purpose of surgical treatment was the release of symptoms, even though the preoperative diagnosis was cardiac myxoma and the patient died from postoperative bleeding. With the improvement of diagnostic procedures and a prolonged life span, the incidence of cardiac metastasis from colorectal cancer is likely to increase. Therefore, to clearly delineate the role of surgical treatment in cardiac metastasis from colorectal cancer, further studies are necessary.

In general, an endocardial myxoma, the most common form of primary cardiac tumor, can be diagnosed by echocardiography, which provides the differential diagnosis of intracavitary atrial echoes, including vegetation, thrombi, and primary or secondary cardiac tumors; however, the detection of cardiac metastasis by echocardiography might be difficult in cases of an infiltrative nature[13,21]. In our case, although the mass was found to have invaded into the right atrium in operative finding, the preoperative echocardiography showed a mobile cardiac mass. Thus, the presumptive diagnosis was atrial myxoma. The differential diagnoses of cardiac myxoma and cardiac metastasis from colorectal cancer might be difficult to determine by echocardiography alone, as in our case, because synchronous cardiac myxoma and colorectal cancer have been reported[22]. Magnetic resonance imaging (MRI) is effective in the evaluation of secondary cardiac tumors, because it can accurately define the pericardium, the myocardial walls, and the cardiac chambers, especially in cases with an infiltrative nature[23,24]. Therefore, in patients with colorectal cancer with a cardiac mass, further diagnostic evaluation such as an MRI, in addition to echocardiography, might be helpful in the differential diagnosis of the cardiac mass.

In conclusion, as reported in autopsy studies, cardiac metastasis from colorectal cancer might not be so rare,

and with improvements in diagnostic procedures, the incidence of cardiac metastasis from colorectal cancer is likely to increase. Thus, in patients with colorectal cancer showing a cardiac mass, further diagnostic evaluation, such as an MRI, might be necessary.

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CASE REPORT

Amelanotic malignant melanoma of the esophagus: Report of two cases with immunohistochemical and molecular genetic study of *KIT* and *PDGFRA*

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Abstract

The author reports herein two cases of amelanotic malignant melanoma of the esophagus. Case 1 is an 87-year-old woman who was admitted to our hospital because of nausea and vomiting. Endoscopic examination revealed an ulcerated tumor of the distal esophagus, and a biopsy was taken. The biopsy showed malignant polygonal and spindle cells. No melanin pigment was recognized. Immunohistochemically, the tumor cells were positive for melanosome (HMB45), S100 protein, KIT and Platelet derived growth factor receptor-α (PDGFRA). The patient was treated by chemotherapy and radiation, but died of systemic metastasis 12 mo after the presentation. Case 2 is a 56-year-old man presenting with dysphagia. Endoscopic examination revealed a polypoid tumor in the middle esophagus, and a biopsy was obtained. The biopsy showed malignant spindle cells without melanin pigment. Immunohistochemically, the tumor cells were positively labeled for melanosome, S100 protein, KIT and PDGFRA. The patient refused operation, and was treated by palliative chemotherapy and radiation. He died of metastasis 7 mo after the admission. In both cases, molecular genetic analyses of *KIT* gene (exons 9, 11, 13 and 17) and *PDGFRA* gene (exons 12 and 18) were performed by the PCR direct sequencing method, which showed no mutations of *KIT* and *PDGFRA* genes. This is the first report of esophageal malignant melanoma with an examination of the expression of KIT and PDGFRA and the mutational status of *KIT* and *PDGFRA* genes.

Key words: Esophagus; Amelanotic melanoma; KIT; Platelet derived growth factor receptor-α

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INTRODUCTION

Primary malignant melanoma of the esophagus is very rare; only case reports and studies of small series have been reported[1-7]. Primary amelanotic malignant melanoma of the esophagus is extremely rare; only a few case reports have been published in the literature[8-13].

Melanoma is a highly aggressive tumor, and *NRAS* and *BRAF* mutations are mainly involved in the pathogenesis of melanoma[14,15]. *KIT* gene, mapped to 4q12, encodes an oncogenic transmembranous receptor tyrosine kinase, KIT, whose ligand is stem cell factor[6-21]. The *platelet derived growth factor receptor-α* (*PDGFRA*) gene, also mapped to 4q12, additionally encodes an oncogenic transmembranous receptor tyrosine kinase, PDGFRA[6-21]. The *KIT* gene plays an important role in melanocyte migration, development, differentiation and tumorigenesis[22]. A few previous studies have shown that activating mutations of the *KIT* gene may lead to tumorigenesis of cutaneous melanoma[14,23]. Since both *KIT* and *PDGFRA* genes are mapped to 4q12, it is anticipated that *PDGFRA* gene mutations are also involved in the tumorigenesis of melanoma, as in the case of gastrointestinal stromal tumors[16-21]. However, the incidence of *PDGFRA* gene mutations in melanoma has rarely been estimated[24].

The author herein reports two cases of esophageal amelanotic malignant melanoma with immunohistochemical and molecular genetic study of *KIT* and *PDGFRA*.

CASE REPORT

Case 1

An 87-year-old woman was admitted to our hospital because of nausea and vomiting. Endoscopic examination revealed an ulcerated tumor of the distal esophagus, and a biopsy was taken. The biopsy was stained with HE. An immunohistochemical analysis was performed, using Dako's Envision method, as previously described[25-30].

Genetic analyses of the *KIT* gene (exons 9, 11, 13 and 17) and the *PDGFRA* gene (exons 12 and 18) were performed by the PCR direct sequencing method, as previously reported[31-35]. The exons of both genes were selected because they are frequent mutation sites[16-21]. The primers are shown in Table 1. In brief, genomic DNA was extracted from paraffin blocks with proteinase K digestion and phenol/chloroform extraction, and subjected to PCR for 40 cycles (94℃ for 1 min, 52℃ for 1 min, 72℃ for 1 min), using a thermal cycler (GeneAmp PCR system 9700, Applied Biosystems, ABI, CA). The annealing temperature was 53℃. PCR products were extracted, and subjected to computed automatic DNA sequencing (ABI PRIZM 3100 Genetic Analyzer, Applied Biosystems, ABI, CA).

The biopsy showed malignant spindle and polygonal cells (Figure 1A and B) suspicious for sarcoma. No melanin pigment was recognized by HE and Masson-Fontana stains. The histology was relatively uniform in the biopsy. Immunohistochemically, the malignant cells were positive for melanosome (Figure 1C) (HMB45, Dako), S100 protein (Figure 1D) (polyclonal, Dako), vimentin (Vim 3B4, Dako), p53 protein (DO-7, Dako), neuron-specific enolase (BBS/NC/VI-H14, Dako), PDGFRA (Santa Cruz, CA, USA), and KIT (polyclonal, Dako) (Figure 1E). The KIT expression was focal. In contrast, the malignant cells were negative for cytokeratins (AE1/3 and polyclonal, Dako), CD3 (M7193, Dako), CD10 (M0727, Dako), CD15 (M0733, Dako), CD30 (M0751, Dako), CD45 (M0855, DAKO), CD45RO (UCHL-1, Dako, CD79α (M7050, Dako), CD20 (L26, Dako), desmin (D33, Dako), α-smooth muscle actin (1A4), CD34 (QBEND10, Dako), chromogranin (DAK-A3, Dako), synaptophysin (polyclonal, Dako), CD56 (MOC-1, Dako), and myoglobin (polyclonal, Dako). Ki-67 labeling (MIB1, Dako) was 80%. A pathologic diagnosis of amelanotic melanoma of the esophagus was made. The molecular genetic analysis showed no mutation of the *KIT* gene (exons 9, 11, 13 and 17) or the *PDGFRA* gene (exons 12 and 18). Examination of the skin, eye and intestine showed no tumors. Therefore, the esophageal melanoma was primary. The patient was inoperative because of weakness and old age, and chemotherapy and radiation were performed. The patient showed systemic metastasis, and died of respiratory failure 12 mo after the first presentation. One additional biopsy of the lung metastasis was obtained, and it showed amelanotic melanoma histology and immunohistochemistry results which were almost the same as those of the primary esophageal biopsy.

Table 1 Primer sequence

	Forward	Reverse
KIT exon 9	5′-TCCTAGAGTAAG CCAGGGCTT-3′	5′-TGGTAGACAGAG CCTAAACATCC-3′
KIT exon11	5′-GATCTATTTTTC CCTTTCTC-3′	5′AGCCCCTGTTTCATA CTGAC-3′
KIT exon 13	5′-GCTTGACATCAG TTTGCCAG-3′	5′-AAAGGCAGCTTG GACACGGCTTTA-3′
KIT exon 17	5′-CTCCTCCAACCT AATAGTGT-3′	5′-GTCAAGCAGAGA ATGGGTAC-3′
PDGFRA exon12	5′-TTGGATATTCAC CAGTTACCTGTC-3′	5′-CAAGGGAAAAGC TCTTGG-3′
PDGFRA exon 18	5′-ACCATGGATCAG CCAGTCTT-3′	5′-TGAAGGAGGATG AGCCTGACC-3′

Case 2

A 56-year-old man presented with dysphagia. Endoscopic examination revealed a polypoid tumor in the middle esophagus. A biopsy was taken (Figure 2A and B). Histologically, the biopsy showed proliferation of malignant spindle cells (Figure 2A and B). No melanin pigment was seen with HE and Masson-Fontana stains. The histology of the tumor was relatively uniform in the biopsy. Immunohistochemically, the tumor cells were positive for melanosome (Figure 2C), S100 protein, vimentin, p53 protein, PDGFRA, and KIT (Figure 2D). The KIT expression was diffuse. In contrast, the malignant cells were negative for cytokeratins, CD3, CD30, CD45, CD45RO, CD79α, CD20, desmin, α-smooth muscle actin, CD34, chromogranin, synaptophysin, CD56, and myoglobin. Ki-67 labeling was 95%. A pathological diagnosis of amelanotic malignant melanoma was made. The molecular genetic analysis identified no mutation of either *KIT* gene (exons 9, 11, 13 and 17) or *PDGFRA* gene (exons 12 and 18). No tumors were identified in the skin, eye and mucosal membrane. Therefore, the esophageal melanoma was considered primary. The patient refused operation, and was treated with palliative chemotherapy and radiation. The patient later showed systemic metastasis and died of melanoma 7 mo after the first presentation. No histological specimens were obtained from the metastatic sites.

DISCUSSION

Amelanotic melanoma of the esophagus is extremely rare, and the pathological diagnosis is very difficult because melanin pigment is absent. In fact, it has been frequently misdiagnosed as other sarcomas[1-13]. Immunohistochemistry of melanoma antigens such as melanosome (HMB45), Malan-A, and S100 protein are mandatory for the diagnosis of amelanotic melanoma. Ultrastructural demonstration of melanosome is also diagnostic. In the present study, both cases were positive for immunoreactive melanosome (HMB45) and S100 protein, confirming the diagnosis. In addition, positive KIT strongly suggests that the tumors are melanomas. Amelanotic melanoma can be diagnosed in biopsy specimens.

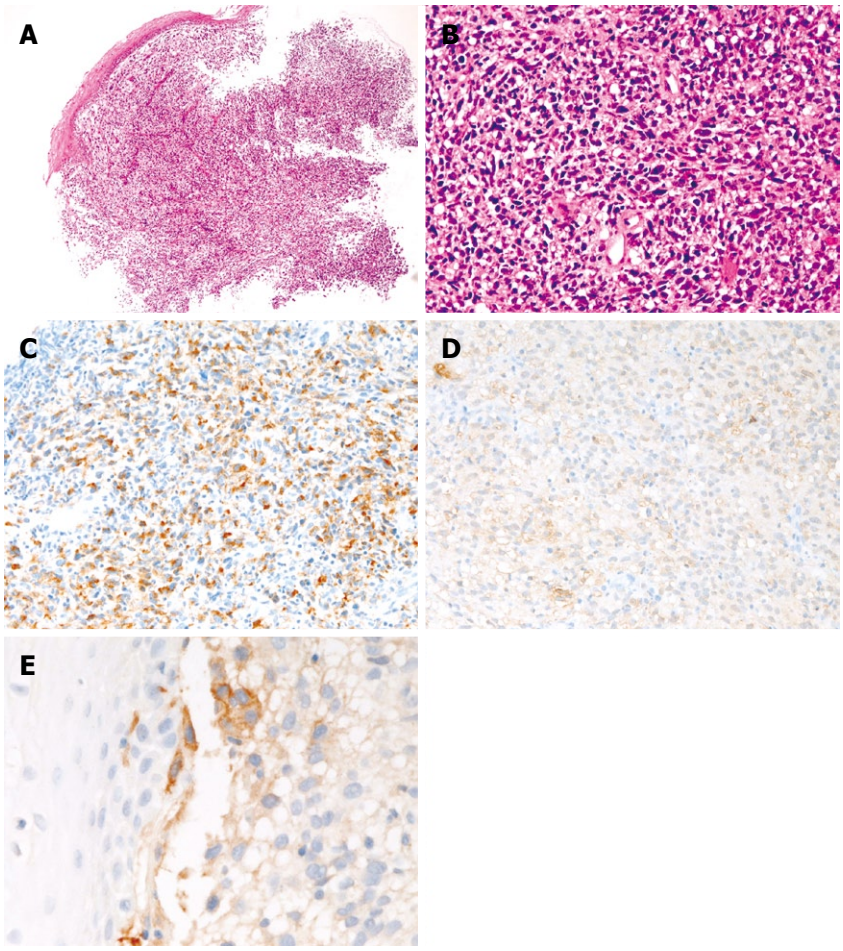

Figure 1 Esophageal amelanotic malignant melanoma in Case 1. A: Low power view of the biopsy (HE, × 20); B: High power view of the biopsy. Malignant polygonal and spindle cells are seen. No melanin pigment is seen (HE, × 200); C: The tumor cells are positive for melanosome (HMB45). (Immunostaining, × 200); D: The tumor cells are positive for S100 protein (Immunostaining, × 200); E: The tumor cells are focally positive for KIT (Immunostaining, × 200).

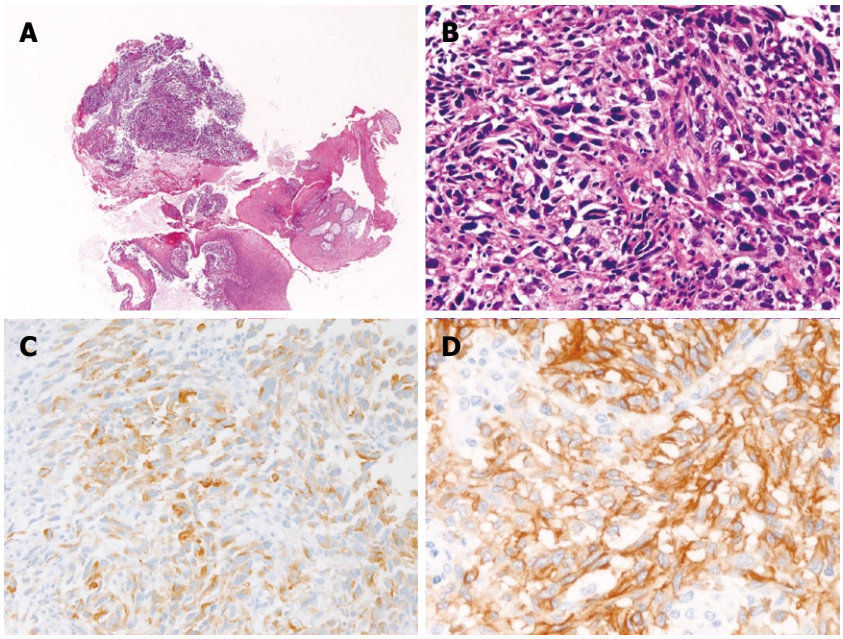

Figure 2 Esophageal amelanotic malignant melanoma in Case 2. A: Low power view of the biopsy (HE, × 20); B: High power view of the biopsy. Malignant spindle cells are seen. No melanin pigment is seen (HE, × 200); C: The tumor cells are positive for melanosome (HMB45) (Immunostaining, × 200); D: The tumor cells are diffusely positive for KIT (Immunostaining, × 200).

Malignant melanoma is a very aggressive tumor irrespective of its location. Malignant melanoma of the esophagus is therefore a very aggressive tumor, and its prognosis is very poor[1-13]. In the present study, the prognosis was indeed very poor in both patients. Operation followed by adjuvant chemotherapy and radiation is the best choice of treatment[1-13]. In the present cases, operation was impossible in one case and was not performed in another case because of the patient's decision not to proceed.

The previously reported cases of esophageal melanoma were only case reports or clinical studies of very small series. There have been no reports of *KIT* and *PDGFRA* expression and mutations in esophageal melanomas. The present study is the first report of esophageal melanoma with an examination of KIT and PDG-

FRA protein expression and gene status of *KIT* and *PDGFRA* in esophageal melanoma. The tumors in the present cases expressed KIT and PDGFRA, but identified no mutations of *KIT* and *PDGFRA*. The positive expressions of KIT and PDGFRA suggest that these transmembranous oncoproteins are present in esophageal melanoma.

In cutaneous melanomas, the percentage of KIT expression varies amongst studies reported by researchers[36]. The percentage of KIT positive cutaneous melanomas in the literature is as follows; 35%[37], 21%[38], 87%[39], 90%[40], 50%[41] and 84%[42]. Sihto *et al*[36] reported that KIT expression in most human solid tumors, including cutaneous melanomas, was due to *KIT* gene amplification. Studies of *KIT* mutations in cutaneous melanoma are scant. Willmore-Payne *et al*[23] showed only 2% of *KIT* mutations in cutaneous melanomas. Sihto *et al*[36] showed no *KIT* mutations in 14 cutaneous melanomas. In contrast, Curtin *et al*[14] showed that *KIT* mutations are present in 39% of mucosal melanomas, in 36% of acral melanomas, in 28% of melanomas on sun-damaged skin, and in 0% of melanomas on non-sun-damaged skin. Beadling *et al*[37] recently reported that *KIT* mutations were present in 23% of acral melanomas, 15.6% of mucosal melanomas, 7.7% of conjunctival melanomas, 1.7% of cutaneous melanoma, and in 0% of choroidal melanomas.

PDGFRA protein expression in melanoma has not been performed, to the best of the author's knowledge. As for *PDGFRA* mutations, Curtin *et al*[24] found no *PDGFRA* mutations in 26 cutaneous melanomas. Sihto *et al*[36] demonstrated no *PDGFRA* gene mutations in 14 cutaneous melanomas.

In summary, the author reported two extremely rare cases of amelanotic malignant melanoma of the esophagus with immunohistochemical and genetic analysis of *KIT* and *PDGFRA*.

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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
London, UK
Gastro 2009 UEGW/World Congress of Gastroenterology
www.gastro2009.org

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 Xiaohua Zazhi* 1999; **7**: 285-287

In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension,

insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 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 billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

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

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EDITORIAL

New perspectives in the treatment of advanced or metastatic gastric cancer

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Abstract

Metastatic gastric cancer remains an incurable disease, with a relative 5-year survival rate of 7%-27%. Chemotherapy, which improves overall survival (OS) and quality of life, is the main treatment option. Meta-analysis has demonstrated that the best survival results obtained in earlier randomized studies were achieved with three-drug regimens containing a fluoropyrimidine, an anthracycline, and cisplatin (ECF). Although there has been little progress in improving median OS times beyond the 9-mo plateau achievable with the standard regimens, the availability of newer agents has provided some measure of optimism. A number of new combinations incorporating docetaxel, oxaliplatin, capecitabine, and S-1 have been explored in randomized trials. Some combinations, such as epirubicin-oxaliplatin-capecitabine, have been shown to be as effective as (or perhaps more effective than) ECF, and promising early data have been derived for S-1 in combination with cisplatin. One factor that might contribute to extending median OS is the advancement whenever possible to second-line cytotoxic treatments. However, the biggest hope for significant survival advances in the near future would be the combination of new targeted biological agents with existing chemotherapy first-line regimens.

Key words: Advanced gastric cancer; Biological agents; Chemotherapy

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INTRODUCTION

Gastric cancer is the fourth most common cancer worldwide, with about 700 000 confirmed deaths annually. Despite an overall global decrease in incidence, gastric cancer is more prevalent in east Asia, eastern Europe, and central and south America than in other countries[1,2].

Chemotherapy is the main treatment option for patients with advanced disease. Median overall survival (OS) of 8-12 mo has been reported in patients undergoing chemotherapy compared with 3-5 mo for those treated with best supportive care[3]. Several drugs have shown good single-agent activity. The response rates range from 10% to 25% and the median duration of response is relatively short. Fluorouracil (5-FU), cisplatin, docetaxel, and, less commonly, paclitaxel, epirubicin, and irinotecan are major components of conventional regimens. Oxaliplatin, capecitabine, S-1, and UFT are also being used in combination chemotherapy. To date, although a large number of trials have been performed, there is no standard treatment for advanced disease.

Intravenous 5-FU remains the most widely used agent and has been the cornerstone of old combination regimens such as FAM (5-FU, doxorubicin, and mitomycin C), FAMTX (5-FU, doxorubicin, and methotrexate), ELF (etoposide, leucovorin, and 5-FU), and ECF (epirubicin, cisplatin, and continuous infusion 5-FU). Although these regimens yield overall response rates (ORRs) of up to 51%, the median survival time in patients with advanced disease has remained irredeemably below 10 mo[4]. Evidently, there is a clear need for more active new agents and regimens.

Combination chemotherapy has been shown to be associated with a statistically significant ($P = 0.001$) survival benefit compared with monotherapy in a meta-analysis of several clinical trials[5]. This corresponded to a small but clinically relevant 1-mo mean average survival benefit. This meta-analysis also evaluated the role of anthracyclines as part of combination chemotherapy. The authors found that including anthracyclines in a 5-FU-cisplatin combination had a modest advantage

over cisplatin-5-FU alone (HR 0.77). Finally, the meta-analysis also showed that three-drug combinations have a significant survival benefit compared with two-drug combinations.

In this context, Van Cutsem *et al*[6] have performed a large-scale random assignment trial comparing the docetaxel, cisplatin, 5-FU (DCF) combination to a control arm of cisplatin plus 5-FU alone (the TAX325 trial). The primary end point of the study was time to progression (TTP) and it was powered to detect an increase in median TTP from 4 mo to 6 mo. The two arms of the study were well balanced for the usual prognostic factors, including weight loss, performance status (PS), and extent of disease. The median TTP was 3.7 mo for patients receiving cisplatin-5-FU, and 5.6 mo for those receiving DCF (HR 1.47; $P = 0.0004$). As a secondary end point, survival was also modestly increased from 8.6 mo for cisplatin-5-FU to 9.2 mo for DCF. The 2-year survival rate was, however, more than doubled for DCF (8.8% for cisplatin-5-FU and 18.4% for DCF). Another measure of efficacy favoring DCF was response rate (37% for DCF, 25% for cisplatin-5-FU). Although this study indicated an efficacy advantage for the three drug combination of DCF, toxicity was also increased and was very substantial. Eighty-one percent of all patients receiving DCF had at least one grade 3 or 4 non-hematologic toxicity, as well as substantially more hematologic toxicity. Despite this, the use of DCF was associated with a better preservation of quality of life and maintenance of clinical benefit compared with cisplatin-5-FU. On the other hand, there was no difference in the treatment-related mortality rate for the two arms. Therefore, like epirubicin, docetaxel, when added to cisplatin-5-FU, produces a modest improvement in efficacy.

ORAL FLUOROPYRIMIDINES

Treatment with oral fluoropyrimidines seems to offer new hope for patients with gastric cancer, which is surprising when only a few years ago, such patients were not thought to be ideal candidates for an oral treatment. In fact, difficulties with the location of primary tumors, surgery (gastrectomy), or presence of metastatic sites affecting intestinal transit (ie. metastases in the peritoneum), made the hypothetic use of an oral treatment a remote possibility. Nonetheless, findings from recent trials that assessed the role of oral fluoropyrimidines seem to place this assumption into a new promising perspective.

Accordingly, capecitabine has been shown to be effective in the treatment of advanced esophagogastric cancer in a phase Ⅲ study comparing capecitabine with fluorouracil and oxaliplatin with cisplatin (the REAL trial)[7]. Patients were randomly assigned to one of four regimens: ECF, epirubicin plus oxaliplatin and 5-FU (EOF), epirubicin plus cisplatin and capecitabine (ECX), or epirubicin plus oxaliplatin and capecitabine (EOX). Comparing ECF to EOF, ECX, and EOX, there were no significant differences in ORRs (41%, 42%, 46%, and 48%, respectively) and grade 3/4 non-hematologic toxicity (36%, 42%, 33%, and 45%, respectively). Non-inferiority in OS was established in both oxaliplatin/cisplatin and capecitabine/5-FU comparisons in this largest randomized controlled trial. Notably, EOX resulted in a significantly improved survival time compared with the control arm ECF. A median survival time of 11.2 mo in the EOX arm was amongst the longest achieved in this setting of patients. Therefore, EOX should now be regarded as one of the standard first-line treatment options for advanced disease. Another trial that compared treatment with 5-FU plus cisplatin with capecitabine plus cisplatin confirmed that efficacy of capecitabine is equivalent to that of 5-FU[8]. Furthermore, although both of these trials were designed to assess whether capecitabine was no worse than 5-FU, the findings generally suggested better outcome in patients who received oral capecitabine.

S-1 is a new oral anticancer drug comprised of tegafur, 5-chloro-2,4-dihydroxypyrimidine, and oteracil potassium. This drug was designed to enhance the efficacy of tegafur, a prodrug of 5-FU. Koizumi *et al*[9] report findings of the S-1 plus cisplatin *vs* S-1 alone for first-line treatment of advanced gastric cancer (SPIRITS trial). Median progression-free survival (6.0 mo *vs* 4.0 mo; $P < 0.0001$) and OS (13.0 mo *vs* 11.0 mo; $P = 0.04$) were significantly longer in the combination group. Response was also significantly improved in patients having target tumors and assigned to S-1 plus cisplatin (54% *vs* 31%). On the basis of these findings, the standard of treatment in Japan has changed, and treatment with combined S-1 plus cisplatin has become the standard of care. The findings from this trial are clearly relevant: for the first time, in a randomized study, the apparently insurmountable wall of 12 mo survival in advanced gastric cancer was crumbled. However, some considerations must be taken into account before combination S-1 plus cisplatin is implemented as standard treatment in western countries. The SPIRITS trial gives no information about the advantage of S-1 over 5-FU when each was combined with cisplatin. The First-Line Advanced Gastric Cancer Study (FLAGS), which compared S-1 with 5-FU, both combined with cisplatin, has responded to this question[10]. This multicenter phase Ⅲ trial has randomized 1053 patients primarily in the USA, Europe, and South America. Median OS was 8.6 mo in the cisplatin/S-1 arm and 7.9 mo in the cisplatin/5-FU arm ($P = 0.1983$). Statistically significant safety advantages for the S-1-based combination were observed regarding the rates of severe neutropenia (18.6% *vs* 40.0%), stomatitis (1.3% *vs* 13.8%), hypokalemia (3.6% *vs* 10.8%), and renal adverse events (all grades: 18.8% *vs* 33.5%). However, this study has not confirmed the efficacy results of the SPIRITS trial in western populations. Thus, the future role of S-1 in gastric cancer could be the inclusion of this oral drug into a three-drug regimen making DCF or ECF better tolerated. What more can we do to increase median OS?

SECOND-LINE CYTOTOXIC TREATMENT

First-line chemotherapy for the treatment of advanced gastric cancer can provide high response rates which are

of a similar magnitude to those achievable with the newer first-line combinations in colorectal cancer. However, a corresponding improvement in the median OS time has not yet been delivered by the currently available gastric cancer regimens. This lack of progress in relation to markedly improving OS times may be a result, in part, of the more limited efficacy of the currently available second- and third-line treatments for advanced disease, although it should be noted that only one third to one half of the gastric cancer patients in clinical studies may actually receive second-line treatments[11]. Data published to date relating to this are restricted to phase Ⅱ studies of small patient populations, and these investigations are therefore not able to provide definitive results. However, second-line treatments are clearly effective to some degree, with ORRs in the region of 11%-32%, median TTP of 2.5-4.5 mo, and median OS times of 5.4-9.3 mo[12-15].

TARGETED BIOLOGICAL AGENTS

The main hope for significant advances in the near future is the combination of new targeted biological agents with existing chemotherapy first-line regimens. A number of different classes of targeted agents have shown promising activity in clinical studies of advanced gastric cancer, including epidermal growth factor receptor (EGFR) and human epidermal growth factor (HER)-2-targeted monoclonal antibodies, antiangiogenic and antiangiogenic/antitumor compounds, and the proteasome inhibitor bortezomib[16-20].

High response and/or disease control rates have been reported for EGFR-targeted cetuximab combined with irinotecan and infusional 5-FU and leucovorin[16-17] and VEGF-targeted bevacizumab combined with irinotecan and cisplatin[19]. In particular, the FOLCETUX study has demonstrated that the addition of cetuximab to the FOLFIRI regimen increased survival in 38 untreated patients with confirmed advanced gastric/gastroesophageal adenocarcinoma. The treatment was delivered for a maximum of 24 wk, and then cetuximab alone was allowed in patients with a complete response (CR), partial response (PR) or stable disease (SD). Consequently, the overall response rate (ORR) was 44% with a CR in four patients and a PR in 11 patients. Sixteen patients had SD. Median expected OS was 16 mo[16]. In another multicenter phase Ⅱ study, 47 patients with metastatic or unresectable gastric cancer received bevacizumab plus irinotecan and cisplatin. With a median follow-up of 12.2 mo among 34 assessable patients, the ORR was 65%, with 20 patients achieving a PR and two patients a CR. Twelve patients had SD. Median survival was 12.3 mo[19].

Trastuzumab exhibits activity in human gastric cancer cells that overexpress HER2/neu. A phase Ⅱ trial has determined the efficacy and tolerability of trastuzumab plus cisplatin in patients with advanced gastric cancer with HER2/neu overexpression/amplification. Preliminary results showed that 6 (35%) out of 17 assessable patients achieved response, 3 (17%) stabilization. There was no grade 4 toxicity[18]. In considering such studies, it is notable that the first-line cytotoxic regimens that have been selected for combination with biological agents tend to be those that are generally considered not to be optimal for the treatment of advanced gastric cancer. This begs the question as to whether the impressive potential of these targeted agents might be more profitably explored in the future within combinations that include standard cytotoxic backbones such as ECF, DCF, EOX, or perhaps S-1 plus cisplatin. Indeed, a number of randomized phase Ⅲ studies incorporating targeted agents in first-line regimens have recently been initiated: the ToGA (Trastuzumab with Chemotherapy in HER2-Positive Advanced Gastric Cancer) study is investigating the effect on progression-free survival of trastuzumab in combination with a fluoropyrimidine plus cisplatin *versus* chemotherapy alone in patients with HER-2-positive advanced gastric cancer, AVAGAST (Avastin® in Gastric Cancer) is investigating OS time in advanced gastric cancer patients receiving either capecitabine and cisplatin plus bevacizumab or chemotherapy alone plus placebo, and the REAL-3 study is investigating the benefit of adding panitumumab to an EOX regimen in patients with locally advanced or metastatic esophagogastric adenocarcinoma.

However, new biological agents could be useful in the management of advanced disease after the failure of first-line treatment. In this context, it is possible that targeted agents may have a future role as single-agent maintenance treatments. Two recent phase Ⅱ studies have pursued this concept[21-22]. A Japanese study has evaluated the activity and safety of everolimus (RAD001), an oral inhibitor of the mammalian target of rapamycin serine-threonine kinase, in 54 pretreated patients with metastatic gastric cancer. At interim analysis, no objective responses were observed but disease control rate was 55% and median TTP was 83 d. Main adverse events were stomatitis (74%), anorexia (51%), fatigue (47%), rash (45%), peripheral edema (23%), thrombocytopenia (21%), diarrhea (19%), pneumonitis (13%), and hyperglycemia (9%). The multicenter AIO phase Ⅱ trial has evaluated tolerability and efficacy of sunitinib in highly pretreated Caucasian patients with unresectable metastatic cancer of stomach, esophagogastric junction or lower esophagus. Among 14 response-evaluable patients, 5 of them showed tumor control for at least 6 wk. With regard to survival, 5 patients experienced early death caused by progressive disease, 7 patients survived > 60 d. Twelve patients were still in follow-up or withdrew informed consent within 60 d after start of therapy. Again, 3 of them survived > 60 d. No serious adverse events occurred.

In conclusion, these studies constitute a potentially important advance, indicating a role for biological agents in the treatment of advanced gastric cancer. Further trials are now needed to clarify their respective position with reference to chemotherapy.

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EDITORIAL

Role of scintigraphy in inflammatory bowel disease

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Abstract

The diagnosis of inflammatory bowel disease (IBD) depends on direct endoscopic visualization of the colonic and ileal mucosa and the histological study of the obtained samples. Radiological and scintigraphic methods are mainly used as an adjunct to endoscopy. In this review, we focus on the diagnostic potential of nuclear medicine procedures. The value of all radiotracers is described with special reference to those with greater experience and more satisfactory results. Tc-99m hexamethylpropylene amine oxime white blood cells remain a widely acceptable scintigraphic method for the diagnosis of IBD, as well as for the evaluation of disease extension and severity. Recently, pentavalent Tc-99m dimercaptosuccinic acid has been recommended as an accurate variant and a complementary technique to endoscopy for the follow-up and assessment of disease activity. Positron emission tomography alone or with computed tomography using fluorine-18 fluorodeoxyglucose appears to be a promising method of measuring inflammation in IBD patients.

Key words: Crohn's disease; Technetium-99m pentavalent dimercaptosuccinic acid; Intestinal inflammation; Scintigraphy; Ulcerative colitis

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INTRODUCTION

The diagnosis and follow-up of patients with inflammatory bowel disease (IBD) is mainly based on endoscopy and the histological study of the obtained biopsy specimens[1,2]. Ileocolonoscopy, gastroscopy and evaluation of small bowel by wireless capsule endoscopy or double balloon enteroscopy offer a successful diagnostic approach in the majority of IBD patients[3,4].

Radiological methods have a secondary role and they are used additionally to endoscopy. They are indicated in cases of suspected complications or small bowel involvement in patients with Crohn's disease (CD)[4-6]. They include conventional radiological methods such as double-contrast barium studies and cross-sectional imaging modalities such as computed tomography (CT), magnetic resonance imaging (MRI), and ultrasound[6-9]. All of them have been proven valuable techniques for evaluation of the effects of the inflammatory process, not only on the bowel wall, but also on other structures within the abdomen[6,8].

Unfortunately, endoscopy as well as the majority of the aforementioned radiological methods are not well tolerated by patients, because of the necessity for adequate bowel preparation and the increased risk of complications, especially when used during the acute phase of bowel inflammation[1,3,8,10].

Alternatively, several studies have demonstrated the reliability of scintigraphic imaging in the diagnosis and assessment of disease activity in IBD. In comparison with other modalities, they are non-invasive techniques and produce no patient discomfort related to instrumentation and preparation, they are not contraindicated in the acute phase and can visualize active disease both in the small and the large bowel[2,11]. Technetium-99m hexamethylpropylene amine oxime labelled white blood cells (Tc-99m HMPAO WBC) have been accepted widely as a reliable method for the diagnosis of IBD, assessment of disease activity

and treatment response[12-14]. Pentavalent Tc-99m dimercaptosuccinic acid [Tc-99m (V) DMSA] seems to be an accurate scintigraphic variant and has been suggested as a complementary technique to colonoscopy for the follow-up and assessment of disease activity[15-17]. Finally, fluorine-18 fluorodeoxyglucose (F-18 FDG) is a promising method for the detection of inflammation in the small and large bowel[18,19].

In this article, we review the current data and future prospects on the role of scintigraphy in diagnosis and evaluation of disease activity in patients with IBD.

THE ROLE OF NUCLEAR MEDICINE IN IBD

Nuclear medicine imaging has played a major role in the diagnosis and detection of inflammation, and has a wide availability of radiotracers. However, its contribution to the localization of small and large bowel pathology in IBD is still under investigation.

Indium-111 oxine labeled leukocytes

Indium-111 (In-111) oxine was the first agent used for *in vitro* leucocyte labeling[2]. The method has been validated by different research groups as a sensitive and specific test for the detection of active intestinal inflammation. However, the high radiation dose, limited availability and poor image quality comprise major disadvantages associated with In-111[2,11,13,14].

Recently, a dedicated whole-body counter has been proposed as an alternative technique to whole-body gamma-camera counting for quantification of disease activity in IBD. It relies on the assumption that all granulocytes migrating into the bowel wall in IBD do in fact end up in the feces, therefore In-111 retention in IBD patients is less compared to that in normal volunteers[20].

Tc-99m HMPAO labeled leukocytes

Tc-99m HMPAO has been used clinically as a cerebral perfusion agent. In 1986, Peters *et al*[21] used it as an alternative to leukocytes labeling and inflammation imaging. Since then, several groups have verified the utility of this imaging technique for IBD. The published data show that it provides a sensitivity of 95%-100%, a specificity of 85%-100% and an accuracy of 92%-100% in the detection, localization and assessment of disease activity. Therefore, its widespread acceptance has been based on the aforementioned favorable results and the advantages of Tc-99m, such as low radiation dose, availability, cost and superior image quality[2,22-24]. It plays an important role in the diagnosis of complications, assessment of disease activity and establishing the extent of small intestine affected. Moreover, it allows a true evaluation of inflammation activity, even during clinical remission or treatment response[2,23,24].

In 2007, Almer *et al*[25] compared leucocyte scintigraphy to intraoperative small bowel enteroscopy and laparotomy findings in CD. They confirmed the reliability of Tc-99m HMPAO WBC in the early diagnosis of small bowel inflammation, and proposed its utility as a first-line investigation modality, especially in children and vulnerable adults.

Despite the wide utility of Tc-99m HMPAO WBC in IBD, controversy still exists about the advantageous imaging time. Early scanning (30-60 min) has been recommended by some authors in order to avoid false positive results caused by intestinal migration of the radionuclide, whereas others favor late scanning because of higher sensitivity. Recently, Sans *et al*[24] have evaluated the optimal scanning sequence for identification of active disease, evaluation of IBD extent, and quantification of disease activity. They reported only slightly lower specificity but higher sensitivity (85% *vs* 100%) and accuracy (85% *vs* 95%) of late scanning (3 h) when compared to early scanning.

Various biomarkers of inflammation have been suggested in selecting patients with suspected IBD for white cell scanning. Given that C-reactive protein constitutes a reliable indicator for the evaluation of inflammatory activity in IBD, patients with $\geqslant$ 5 mg/L should be selected for white cell scanning in order to reduce the number of false negative results[26].

Alonso *et al*[27,28] have applied Tc-99m HMPAO WBC to patients with subclinical gut inflammation. This group studied patients with seronegative spondyloarthropathy without clinical evidence of IBD. They confirmed the utility of the method in the assessment of bowel inflammation, even if it remains subclinical. Moreover, they described a possible role of labeled leukocytes in identifying the patients who are suitable for therapy with sulfasalazine, and in assessing treatment effectiveness and disease relapse[27,28]. El Maghraoui *et al*[29] have certified the aforementioned results and demonstrated a statistically significant correlation between Tc-99m HMPAO-labeled leukocytes and ileocolonoscopy.

The usefulness of this technique in early detection of postoperative asymptomatic recurrence of CD has been suggested. Biancone *et al*[30] have demonstrated that, in patients with CD who had an ileocecal resection in the previous 6 mo, the perianastomotic 30 min Tc-99m HMPAO WBC uptake was significantly associated with disease recurrence.

The role of Tc-99m HMPAO-labeled leukocytes single photon emission computerized tomography (SPECT) in IBD has also been investigated. SPECT images provide accurate assessment of inflammation in both the small and large bowel and precise anatomical details of CD lesions. Moreover, they are independent of bone marrow activity, thus allowing detailed disease evaluation within the pelvis[31,32].

The aim of several groups has been to evaluate and compare the diagnostic accuracy of Tc-99m HMPAO-labeled leukocytes and CT in IBD. They have demonstrated the superiority of scintigraphy in detecting segmental inflammatory activity and proximal extension of bowel involvement. CT displays excellent suitability for the localization of fibrostenotic bowel disease and the recognition of complications, but has a four-fold higher radiation exposure[33-35].

Several studies have supported the utility of Tc-

Table 1 Summary of published studies evaluating the use of Tc-99m HMPAO WBC in IBD

Study	*n*	Study design	Sensitivity	Specificity
Adult population				
Sciarretta *et al*[23]	103	Known active CD compared with colonoscopy	95%	100%
Mairal *et al*[50]	27	Known IBD compared with In-111 HIG	100%	85%
Giaffer *et al*[13]	31	Suspected IBD compared with In-111 oxine labeled leukocytes	85% at 40 min 94% at 120 min	87% at 40 min 71% at 120 min
Kolkman *et al*[33]	32	Known IBD compared with CT	79% for CD 81% for UC	98% for CD 86% for UC
Molnár *et al*[34]	28	Known active CD compared with spiral CT	76.1%	91%
Almer *et al*[25]	48	Known active CD with small bowel inflammation compared with intraoperative small bowel enteroscopy and laparotomy findings	85%	81%
Pediatric population				
Charron *et al*[40]	215	Acute intestinal inflammation in patients with and without IBD	90%	97%
Cucchiara *et al*[38]	48	Suspected IBD compared with colonoscopy	76.2%	NA
Charron *et al*[42]	130	Exclude inflammation in suspected IBD compared with colonoscopy	94%	99%
Alberini *et al*[37]	28	Known IBD compared with endoscopy, ultrasonography and contrast radiology	75%	92%
Charron *et al*[35]	313	Known IBD compared with colonoscopy	92%	94%

NA: Not applicable; CD: Crohn's disease; CT: Computed tomography; IBD: Inflammatory bowel disease; UC: Ulcerative colitis.

99m HMPAO WBC in pediatric patients with IBD. They have suggested that labeled leukocytes cannot replace endoscopy for initial diagnosis but they do have a place in the decision for colonoscopy[36,37]. Patients with negative Tc-99m HMPAO WBC scans may avoid unnecessary colonoscopy. However, Cucchiara *et al*[38], after evaluating 48 children, have concluded that a positive test indicates the presence of inflammation but a negative result does not rule out inflammation, since the technique may miss cases with mild disease.

Moreover leukocytes scintigraphy can be considered a reference method for clarifying the extent of inflammation when colonoscopy is not completed successfully, or the findings in contrast radiography are negative[36,37,39]. Although SPECT allows the identification of additional involved segments over planar images, its performance in children seems to be rather difficult[37,40].

The accuracy of Tc-99m HMPAO WBC in differentiating continuous from discontinuous colitis has also been examined[37,41]. In 77 children with active CD, discontinuous uptake was revealed in 63, and among 29 children with ulcerative colitis (UC), continuous uptake was revealed in 23[41]. It should also play an important role in the follow-up of patients and it could be used as a diagnostic tool for assessing recurrence or response to therapy, thus reducing the need for repeated colonoscopy[36,37].

In a report by Charron *et al*[35], the accuracy of CT and Tc-99m HMPAO WBC scintigraphy versus colonoscopy in IBD has been compared. After evaluating 313 consecutive children who underwent a labeled leucocyte test and comparing with colonoscopy, the sensitivity of scintigraphy was 92%, specificity was 94%, positive predictive value was 96%, negative predictive value was 93% and accuracy was 94%. Tc-99m HMPAO WBC scan is unlikely to miss significant inflammation, while CT has lower sensitivity for detecting inflammation in the bowel wall. However, similar to the adult population, the incidence of complications detected by CT is higher than with scintigraphy[35].

Compared to other modalities, Tc-99m HMPAO WBC scintigraphy is non-invasive, practical, safe, rapid and has excellent diagnostic sensitivity (Figure 1). It requires no bowel preparation, causes no discomfort and exposes patients to less radiation, namely the effective radiation dose for Tc-99m HMPAO WBC imaging is 3 mSv, for barium small bowel follow-through, 6 mSv, and for barium enema, 8.5 mSv[36,39,40,42]. Additional important advantages are the ability to evaluate the small and the large bowel simultaneously and the superiority over small bowel follow-through and CT, in the initial screening and detection of inflammation in patients with IBD[35,36,42]. A concise form of the published data is presented in Table 1.

The high cost, time-consuming *in vitro* labeling procedure, radiation microdosimetry, as well as the handling and reinjection of blood constitute the main shortcomings of the procedure when compared to other scintigraphic modalities.

Tc-99m (V) DMSA

Tc-99m (V) DMSA is a tumor-seeking agent of low molecular weight developed in 1981. It has been used successfully in the scintigraphic diagnosis of various malignant tumors[43-47]. Moreover, it has been proven advantageous in the diagnosis of inflammatory diseases such as osteomyelitis, psoas muscle abscess, and bone and joint infection[48,49].

The mechanism of Tc-99m (V) DMSA localization in tumors and inflammation remains unclear. In some cases, it resembles the phosphate ion because it accumulates in lesions where calcification is present. However, the increased capillary permeability followed by infiltration of the radiotracer into the interstitial space seems to be the most probable mechanism of uptake in inflammatory lesions[16,17,48,49].

Tc-99m (V) DMSA scintigraphy requires no bowel preparation, no blood manipulation and causes no patient discomfort. Moreover it has a low cost, ideal physical characteristics, and simple preparation procedure from cold kits[15-17]. Its utility in the diagnosis

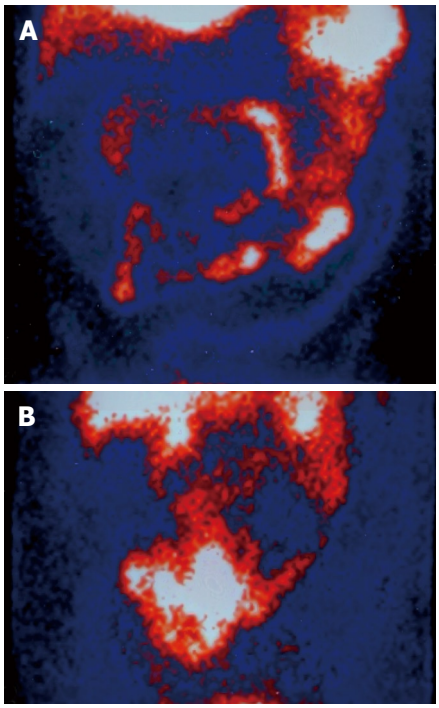

Figure 1 Tc-99m HMPAO WBC scintigram. A: Ulcerative colitis (UC) with intense inflammation of the entire large bowel; B: Crohn's disease (CD) with intense inflammation in the small bowel and the descending colon.

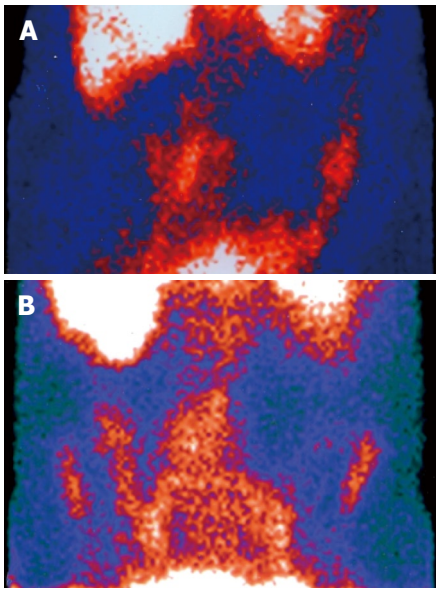

Figure 2 Tc-99m (V) DMSA scintigram. A: UC with intense inflammation mainly in the transverse and the descending colon; B: CD with intense inflammation of the terminal ileum and the ascending colon.

of inflammation combined with the aforementioned advantages have given a new impulse to research groups to evaluate its role in IBD.

In 2001, Lee *et al*[15] appraised the potential use of Tc-99m (V) DMSA scintigraphy in the detection and localization of intestinal inflammation. The study enrolled 62 patients with suspected intestinal inflammation, namely IBD, appendicitis, and antibiotic-associated, infective, eosinophilic and ischemic colitis. The scintigraphic findings were compared to colonoscopy and biopsy results. The overall sensitivity was 95%, specificity, 94%, and accuracy, 95%. The three false negative cases were attributed to a mild degree of inflammation. Findings were false positive in two cases as a result of coexisting active bleeding from the gastrointestinal tract and colonic adenocarcinoma, seen at colonoscopy with biopsy[15].

In 2003, Koutroubakis *et al*[16] evaluated the use of Tc-99m (V) DMSA for the assessment of disease activity in patients with IBD. They examined three groups of patients. The first group enrolled 36 patients who had an exacerbation of previously demonstrated disease or had a first attack of IBD. The second group included 28 patients who were in remission from IBD. The third group included 12 patients with miscellaneous bowel disease, namely ischemic, infectious or segmental colitis associated with diverticulosis. The scintigraphic findings were compared to colonoscopy and biopsy results. In the detection of active disease, the sensitivity was 92%, the specificity was 86%, the negative predictive value was 85.1% and the positive predictive value was 91.9%. A high correlation of the scintigraphic activity index with the endoscopic and histological inflammatory activity was found. Findings were false negative in three cases with active disease because of a mild degree of inflammation[16].

A direct comparison of Tc-99m (V) DMSA with Tc-99m HMPAO WBC scintigraphy in the evaluation of IBD has been undertaken by Stathaki *et al*[17] in 2008. The favorable results of the two previous studies, in combination with the advantages of the method, could establish it as an ideal alternative scintigraphic method. The study enrolled 23 patients who had an exacerbation of previously proven IBD or a first attack of the disease. Tc-99m (V) DMSA scintigraphy was performed after clinical and endoscopic confirmation of active disease and true positive labeled leucocyte scintigraphy. The full agreement among the scintigraphic modalities was 72.5%. The agreement among endoscopy and scintigraphy was 91.9% and 84.4% for Tc-99m HMPAO WBC and Tc-99m (V) DMSA, respectively. The overall sensitivity was 91% for Tc-99m HMPAO WBC and 84% for Tc-99m (V) DMSA. False negative results for Tc-99m (V) DMSA scintigraphy were seen in two patients with UC, probably because of a mild degree of bowel inflammation[17].

Data suggest that Tc-99m (V) DMSA scintigraphy provides a useful approach in the diagnosis of active disease and assessment of disease activity (Figure 2). Despite that, it cannot replace Tc-99m HMPAO WBC for the evaluation of disease localization. Probably, it is not the ideal method for the diagnosis of IBD but it has a place in the follow-up and assessment of disease activity, progression and treatment response[17]. A concise form of the published data is presented in Table 2.

Other radiotracers

A variety of radionuclides has been applied to IBD investigation. Some of them have not gained widespread clinical use because of limitations and disappointing results, while others seem to have a definite role.

Table 2 Summary of published studies evaluating the use of Tc-99m (V) DMSA in IBD

Study	*n*	Study design	Sensitivity (%)	Specificity (%)
Lee *et al*[15]	62	Intestinal inflammation compared with colonoscopy	95	94
Koutroubakis *et al*[16]	76	Active and inactive IBD compared with colonoscopy	92	86
Stathaki *et al*[17]	23	Active IBD compared with Tc-99m HMPAO WBC and colonoscopy	84	NA

In-111 or Tc-99m human polyclonal immunoglobulin (HIG) has been used in the diagnosis of inflammation and it has been evaluated in IBD. Comparative studies have demonstrated sensitivity, specificity and accuracy of 100%, 85% and 96%, respectively, for labeled leukocytes and 70%, 85% and 74% for In-111 HIG[50]. Tc-99m HIG scintigraphy had 33% sensitivity while Tc-99m HMPAO WBC imaging had 100% sensitivity in the detection of active IBD[51]. On the basis of these results, the role of In-111 HIG is confined to the diagnosis of inflammation only when there is no other alternative modality[50]. On the other hand, Tc-99m HIG has no role in the evaluation of patients with IBD[51].

In vivo specific labeling of granulocytes using Tc-99m labeled anti-granulocyte monoclonal antibodies (AGAb) comprises a different approach. They do not require leucocyte isolation, are stored as cold kits and can selectively label granulocytes. Different AGAb have been designed, among them BW 250/183 and Leukoscan[52-55]. Tc-99m BW 250/183 was found to be inferior to Tc-99m HMPAO WBC in the detection of small bowel involvement, although the accuracy between the two scintigraphic methods for the localization of disease in the large bowel was comparable[52]. With respect to Tc-99m Leukoscan, its diagnostic value in IBD is low[53,54]. However, a recent study by Kerry *et al*[55] has found that Tc-99m Leukoscan has higher sensitivity and specificity at 2 h (44% and 100% respectively) and 4 h (75% and 50% respectively) planar imaging compared to that in previous publications. SPECT images at 4 h showed additional areas of uptake, raising the sensitivity to a value similar to that of Tc-99m HMPAO WBC, namely 88%. Although sensitivity is high, the low specificity limits its application for the investigation of IBD[55].

Research groups have evaluated the role of AGAb imaging in pediatric patients with IBD. Bruno *et al*[56] have found that the overall sensitivity of Tc-99m BW 250/183 was 94% for CD and 85% for UC. Sensitivity of scintigraphy compared to colonoscopy, radiology and ultrasonography was 90%, 76%, 75% and 55%, respectively. However, it did not appear sufficiently specific in identifying clinical remission, probably because of the presence of tissue inflammation in about 50% of biopsy samples, although patients were considered to be in clinical remission and with negative colonoscopy. The authors have recommended Tc-99m BW 250/183 as a useful tool in the detection of intestinal inflammation in children and young patients with IBD. However, because of its low specificity, endoscopic and histological confirmation is mandatory for all positive cases[56]. The efficacy of Tc-99m Leukoscan has been evaluated in a small series of pediatric patients with IBD. The reported sensitivity per patient was 90% and per bowel segment, 57%. The latter was improved with the use of SPECT[57].

In 1984, Hanna *et al*[58] worked on the labeling of leukocytes with Tc-99m stannous colloid, and reported the clinical application of this new imaging modality in IBD. Despite its usefulness as an alternative when other agents are not available, the activation of leukocytes, which reduces the *in vivo* viability, constitutes a shortcoming[59]. Recently, its use in the initial evaluation of children with suspected IBD has been assessed. The combination of the reported results (sensitivity 88%, specificity 90%) and the aforesaid advantages support its utility in the initial assessment of childhood IBD[59].

The primary data on the role of In-111 anti E-selectin monoclonal antibodies are encouraging, given that it can identify areas of inflammation in CD and UC. Still, they are not supported sufficiently to gain acceptance in the field of IBD[60].

Positron emission tomography (PET)

PET with F-18 FDG is a functional imaging modality which identifies areas of increased glucose metabolism. It has been found to be effective in the evaluation of malignancies, inflammation and infection. Preliminary studies have shown favorable results in the assessment of disease activity in IBD[19].

In a small study of four patients with CD and two with UC, PET scanning demonstrated high radionuclide uptake in the inflamed segments, which had been detected on endoscopy and confirmed by histology. The potential utility of this non-invasive modality, as well as its usefulness for follow-up was suggested[61]. Neurath *et al*[18] have compared F-18 FDG, hydro-MRI and granulocyte scintigraphy with labeled antibodies (Tc-99m BW 250/183) in the detection of disease activity in 59 patients with CD. The sensitivity and specificity reported for F-18 FDG was 85% and 89%, for hydro-MRI, 67% and 93%, and for Tc-99m BW 250/183, 41% and 100%. It appears to be an accurate modality for detecting inflammation, considering that it allows a simultaneous non-invasive analysis of affected segments in both small and large bowel. Moreover, it is helpful in evaluating possible inflammatory activity in detected stenosis, which is important for its therapeutic application[18].

Recent studies have assessed the role of PET in the investigation of pediatric IBD. It diagnosed active disease in 80% of childhood cases with known IBD, and F-18 FDG uptake correlated with the endoscopic findings in 83.8% of the patients. PET recognized diseased segments that were not detected by other diagnostic methods, probably because of the limited accessibility at endoscopy. Moreover, it is the least invasive technique, can provide additional information to the diagnostic data obtained by other modalities, and exposes patients to

lower radiation doses[62]. Löffler *et al*[63], using histology as a reference standard, reported F-18 FDG PET sensitivity, specificity and accuracy to be 98%, 68% and 83%, respectively, for large bowel, and 100%, 86% and 90% for small bowel involvement. Based on these favorable results, the authors have recommended the inclusion of PET in the initial investigative algorithm for the evaluation of bowel inflammation and treatment response. On the other hand, its moderate specificity renders indispensable the endoscopic and histological confirmation of all positive cases[63].

Coupling CT to PET combines the functional data obtained from PET with the anatomical data provided by CT. Its role in the detection and localization of disease activity in IBD has been evaluated. In a pilot study, Meisner *et al*[64] have validated the results of previous reports concerning the role of PET in IBD, and they have investigated the use of sequential CT. In most cases, the simultaneous transaction of CT was not essential but it allowed better anatomical analysis in patients who had been surgically treated, and in those with inflammation of the small bowel. There was a high correlation between PET activity and disease activity, as determined by other currently used modalities[64].

Louis *et al*[65] have similarly concluded that coupling PET with CT allows a more accurate anatomical identification and evaluation of F-18 FDG uptake, and it gives more morphological information, namely, the presence of strictures. The technique can detect almost all bowel segments with moderate and severe lesions and a significant proportion with only mild lesions. Of great scientific interest were the combined findings of increased F-18 FDG uptake and bowel wall thickening in PET/CT, which were observed in some segments without endoscopic evidence of lesions. One explanation might be the detection of active disease deeper in the bowel wall, which is an additional benefit of this diagnostic modality[65].

Recently, the role of PET/CT in patients with UC in remission has been evaluated. Although clinical remission was strictly defined, four out of the 10 patients who participated in the study had increased F-18 FDG uptake in the colon. This may be explained by the presence of asymptomatic inflammation, attributed to chronic low-grade activity or to the succession of flare and quiescence. The possibility of representing a normal variant or a false positive result could not be excluded. This finding necessitates further understanding of disease remission. The authors have suggested that PET/CT is a highly sensitive method, however, future studies will define its precise role among all available diagnostic modalities in disease evaluation and treatment monitoring[66].

At present, published data have suggested a high diagnostic value of F-18 FDG PET alone or PET/CT in adult and pediatric patients with IBD. However, the physiological distribution of the radionuclide, mainly in the urinary tract, and to a minor degree in the gastrointestinal tract, may compromise abdominal PET imaging of patients with IBD. In order to avoid any false results, the utility of quantitative analysis using the standardized uptake value (SUV) has been suggested. A cutoff RSUV (ratio between SUV of inflamed bowel and SUV of liver) value of 1.47 seems to be reliable for the identification of areas with significant bowel inflammation[19]. Recently, various methods of labeling leukocytes with F-18 FDG have been reported. F-18 FDG WBC are taken up in the reticuloendothelial tissue and follow the normal leukocyte distribution *in vivo*. Its role as a method for non-invasive quantification of IBD has been evaluated mainly in animal models[67]. The localization of the inflammatory process and the degree of tracer uptake are correlated with the endoscopic and histological findings. In the future, the method may be useful in determining the cause of pathological abdominopelvic tracer uptake, namely, inflammation *versus* malignancy. These are preliminary results that require further investigation in humans[67].

CONCLUSION

In this review, we have presented the role and the future prospects of nuclear medicine in IBD. Although it has no primary role in the diagnosis, it might be considered when colonoscopy is not completed successfully or other imaging modalities are negative. However, its contribution to the assessment of disease extent and activity, monitoring treatment response, and differentiating between active CD and UC is well established. Tc-99m HMPAO WBC have gain widespread clinical use while Tc-99m (V) DMSA seems to provide an accurate scintigraphic variant and a complementary technique to colonoscopy for follow-up and assessment of disease activity. The preliminary results on the role of F-18 FDG PET or PET/CT in the diagnosis and follow up of patients with IBD are encouraging. F-18 FDG WBC seem to be a promising future prospect, given that they can differentiate between the cause of pathological tracer uptake, namely, inflammation *versus* malignancy. Further investigation is essential in order to verify all the aforementioned favorable preliminary results.

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REVIEW

Helicobacter pylori infection and endocrine disorders: Is there a link?

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Abstract

Helicobacter pylori (*H pylori*) infection is a leading world-wide infectious disease as it affects more than half of the world population and causes chronic gastritis, peptic ulcer disease and gastric malignancies. The infection elicits a chronic cellular inflammatory response in the gastric mucosa. However, the effects of this local inflammation may not be confined solely to the digestive tract but may spread to involve extra-intestinal tissues and/or organs. Indeed, *H pylori* infection has been epidemiologically linked to extra-digestive conditions and diseases. In this context, it has been speculated that *H pylori* infection may be responsible for various endocrine disorders, such as autoimmune thyroid diseases, diabetes mellitus, dyslipidemia, obesity, osteoporosis and primary hyperparathyroidism. This is a review of the relationship between *H pylori* infection and these endocrine disorders.

Key words: *Helicobacter pylori*; Hormones; Thyroid; Osteoporosis; Diabetes; Dyslipidemia

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INTRODUCTION

Helicobacter pylori (*H pylori*) is a gram-negative, spiral-shaped pathogenic bacterium that specifically colonizes the gastric epithelium and causes chronic gastritis, peptic ulcer disease and/or gastric malignancies[1,2]. The infection induces an acute polymorphonuclear infiltration in the gastric mucosa. If the infection is not effectively cleared, this acute cellular infiltrate is gradually replaced by an immunologically-mediated, chronic, predominantly mononuclear cellular infiltrate[3]. The latter is characterized by the local production and systemic diffusion of pro-inflammatory cytokines[4], which may exert their effect in remote tissues and organic systems[5]. As a result, *H pylori* infection has been epidemiologically linked to some extra-digestive conditions, including endocrine disorders (Table 1), although there are contradictory data regarding the relationship between *H pylori* infection and these diseases.

H pylori AND DIABETES MELLITUS

The relationship between diabetes mellitus (DM) and *H pylori* infection is controversial. According to some studies there is a high prevalence of *H pylori* infection in patients with either type I [6-9] or type II DM[10-13] which is correlated with the duration of DM[7,9], the presence of dyspeptic symptoms[13,14] and cardiovascular autonomic neuropathy[13,15], age[6,8], gender[16], body mass index (BMI)[16], blood pressure[16], fasting glucose[16] and the HbA1c levels[16]. In particular, the prevalence of *H pylori* infection was found to be higher in obese, female, middle-aged patients with a long standing DM, dyspeptic symptoms, cardiovascular autonomic neuropathy and increased blood pressure, fasting glucose levels and HbA1c values[6-9,13-16]. This could be related to a reduced gastric motility and peristaltic activity[10], various chemical changes in gastric mucosa following non-enzymatic glycosylation processes[10] and an impaired non-specific immunity observed in diabetic patients[11].

In contrast, other studies showed that *H pylori* infection is not associated with DM, as there is no

difference in the prevalence of *H pylori* infection between diabetics and non-diabetics[17], regardless of the type[8,17-22] and duration of DM[18,19,22] and/or severity of dyspeptic symptoms in patients with DM[22]. The presence of micro-angiopathy in patients with DM may be a negative factor for colonization by *H pylori*, because micro-vascular changes in the gastric mucosa may create an unfavourable environment for the establishment or survival of *H pylori*[16]. Interestingly, one study even showed a lower sero-prevalence of *H pylori* in patients with DM, in comparison with the healthy population[23], while another showed a significantly lower incidence of *H pylori* infection in diabetics with active duodenal ulceration, as compared with non-diabetics[24].

The relationship between gastrointestinal symptoms in DM and *H pylori* infection is also controversial. According to some studies, there is no difference between diabetics and non-diabetics concerning the prevalence of *H pylori*-related gastro-duodenal disorders[17]. Moreover, *H pylori* infection was not associated with either delayed gastric emptying[9,25] or upper gastrointestinal symptoms in DM[19,21,25]. On the other hand, a high prevalence of esophagitis and peptic ulcer was found in *H pylori*+ve patients with DM, with or without dyspepsia, especially those with cardiovascular autonomic neuropathy[13,15] suggesting that this population should be considered as "high risk" for *H pylori* infection and suitable candidates for treatment[12]. In addition, some data demonstrated a higher prevalence of *H pylori* infection in diabetic patients with dyspepsia[14,26], reactive gastritis[27] and chronic gastritis[26] compared to those with no signs or symptoms of gastrointestinal disease.

The relationship between DM complications and *H pylori* infection is another issue which is contentious and deserves further investigation, as only few data are available. According to some data there is no relationship between *H pylori* infection and diabetic complications, such as nephropathy[13], retinopathy[13], and/or micro-angiopathy[16] while other data shows that virulent strains of *H pylori*, such as cytotoxin-associated gene $CagA^+$, are associated with macro-angiopathy[16], neuropathy[16] and micro-albuminuria in type Ⅱ diabetic patients, maybe due to an immuno-mediated injury at the level of the endothelium, caused by a systemic immune response to the infection, leading to albumin leakage[28]. Additionally, some data indicate a possible association of *H pylori* infection and the development of coronary heart disease, thrombo-occlusive cerebral disease, or both, in diabetic patients[29].

One point on which all studies seem to converge is that the effectiveness of eradication regimens for *H pylori* infection is significantly lower in diabetics than in non-diabetics[20,30-32] whereas re-infection rates seem to be higher, especially in patients with type Ⅱ DM compared to the general population[20,31]. This may be due to changes in the gastric microvasculature leading to reduced absorption of antibiotics. Alternatively, frequent antibiotic use in diabetics may result in the development of resistant *H pylori* strains[30,32]. Moreover, type I diabetic patients achieve lower *H pylori* eradication rates on standard triple therapy than non-insulin-dependent diabetic subjects, regardless of the dosage and/or the duration of therapy[20,31,32], and higher re-infection rates one year after eradication of *H pylori* compared with control subjects[33]. Quadruple therapies seem to cure a large percentage of patients who fail first-line therapy, although this is accompanied by a greater incidence of minor side effects[20,31]. These data suggest that vaccine development seems to be the only effective long term treatment for patients with DM[20].

Noteworthy is the observation that children with type I DM and *H pylori* infection had an increased daily insulin requirement compared with their uninfected peers[34]. Finally, several issues, such as the role of *H pylori* in etiopathogenesis of DM and the influence of *H pylori* eradication on the control of DM, remain to be elucidated.

Table 1 Endocrine disorders in relationship with *H pylori* infection

Endocrine disorders
Autoimmune thyroid diseases
Autoimmune atrophic thyroiditis
Hashimoto's thyroiditis
Thyroid mucosal associated lymphocyte tissue (MALT) lymphoma
Diabetes mellitus
Dyslipidemia
Obesity
Osteoporosis
Primary hyperparathyroidism

H pylori AND OSTEOPOROSIS

There are limited data regarding the association between *H pylori* infection and osteoporosis. According to one study, *H pylori* infection was not accompanied by significant changes in levels of markers of bone metabolism in children, such as estradiol, parathyroid hormone (PTH), cross-linked collagen I carboxy terminal telopeptide, total alkaline phosphatase (ALP), bone-specific ALP, N-terminal cross-links of human pro-collagen type I , osteocalcin, calcium and phosphate[35]. In another study, infection by $CagA^+$ *H pylori* strains was more prevalent in men with osteoporosis compared to the general population, who showed reduced systemic levels of estrogens and increased bone turnover[36]. *H pylori* infection by $CagA^+$ strains may therefore be considered a risk factor for osteoporosis in men[36]. Further studies are required to clarify the relationship between *H pylori* infection and osteoporosis and whether *H pylori* infection causes time-dependent changes in bone turnover markers during the long course of this chronic inflammatory disease.

H pylori AND HYPERPARATHYROIDISM

There are only a few studies attempting to clarify the association between *H pylori* infection and hyperparathyroidism. In fact, only one study showed that *H pylori* infection was more prevalent amongst patients with primary hyperparathyroidism (PHPT) than in the

general population, suggesting that patients with PHPT, and especially those with dyspeptic symptoms, should be evaluated for *H pylori* infection and treated appropriately if positive[37]. Also, a case report described an association of PHPT with duodenal ulcer and *H pylori* infection[38]. On the other hand, another study claimed no significant relationship between parathyroid abnormalities and *H pylori* infection in haemodialysis patients and this study found that a longer period of dialysis therapy was related to a decreased ability of these patients to produce antibodies against *H pylori*[39].

H pylori AND OBESITY

The relationship between obesity and *H pylori* infection is controversial. According to some studies, the risk of *H pylori* infection does not increase in overweight young persons[40] and *H pylori* seropositivity or CagA antibody status are not associated with the BMI[41,42] or fasting serum leptin levels[41]. Furthermore, one study indicated an inverse relationship between morbid obesity and *H pylori* seropositivity, leading to the hypothesis that the absence of *H pylori* infection during childhood may enhance the risk of the development of morbid obesity[43]. In contrast, other studies showed that obesity[10] and/or an elevation of the BMI[44] may be associated with an increased incidence of *H pylori* colonization, probably as a result of reduced gastric motility[10]. In addition, the incidence of *H pylori* infection in patients undergoing Roux-en-Y gastric bypass surgery for morbid obesity was higher than that found in all patients undergoing endoscopies and biopsy, even though the incidence of infection was not higher in controls matched for age[45].

The relationship between obesity and *H pylori* eradication is also controversial. There are data which demonstrate that eradication of *H pylori* significantly increases the incidence of obesity in patients with peptic ulcer disease, since it increases the level of BMI[46,47], and/or enhances the appetite of asymptomatic patients, due to an elevation of plasma ghrelin[48] and a reduction of leptin levels[49,50]. In fact, *H pylori* infection caused a marked reduction in plasma levels of ghrelin[44,49,51-53], as a result of a negative effect of this infection on the density of gastric ghrelin-positive cells[51,54] and an increase in plasma levels of leptin and gastrin[49,55,56]. Since ghrelin exerts orexigenic and adipogenic effects in contrast to leptin which exerts anorexigenic effects[52], alterations in plasma levels of gastric originated appetite-controlling hormones in children and adults infected by *H pylori* may contribute to chronic dyspepsia and loss of appetite[49]. Consequently, *H pylori* can be a "protective" factor against the development of becoming overweight[50]. In contrast, other studies showed that there are no differences in plasma ghrelin levels between *H pylori*+ve and *H pylori*-ve patients matched for age and BMI[57] and that successful eradication of *H pylori* had no effect on plasma ghrelin levels[44,57].

H pylori AND THYROID DISEASES

There have been controversial reports linking *H pylori* infection to thyroid disorders including autoimmune thyroid disorders (ATD) such as autoimmune atrophic thyroiditis[58] and Hashimoto's thyroiditis[59], or thyroid mucosal associated lymphocyte tissue (MALT) lymphoma[60].

Thus, some studies have reported an increased prevalence of *H pylori* infection in adults[58,61,62] and children[63] with ATD and a relationship between *H pylori* infection and the presence of high titers of thyroid auto-antibodies, such as anti-thyroglobulin (anti-Tg) and anti-thyroperoxidase (anti-TPO) antibodies[58,61,62] resulting in abnormalities of gastric secretory function[58]. It has also been suggested that CagA$^+$ *H pylori* strains increase the risk for ATD, especially in women, and that they are involved in the pathogenesis of Hashimoto's thyroiditis. This is based on the detection of monoclonal antibodies against Cag-A$^+$ *H pylori* strains which cross-react with follicular cells of the thyroid gland and also on the fact that *H pylori* strains possessing the Cag-A pathogenicity island carry a gene encoding for an endogenous peroxidase[61]. Moreover, the strong correlation between IgG anti-*H pylori* antibodies and thyroid auto-antibodies, as well as the observation that eradication of *H pylori* infection is followed by a gradual decrease in the levels of thyroid auto-antibodies[64], suggest that *H pylori* antigens might be involved in the development of autoimmune atrophic thyroiditis or that autoimmune function in this disease may increase the likelihood of *H pylori* infection[58]. One study showed a significant decrease of Free-T_3 and Free-T_4 in *H pylori*+ve subjects compared to *H pylori*-ve controls[62].

On the contrary, other studies showed no differences in the serum levels of thyroid hormones or thyroid auto-antibodies in patients with and without *H pylori* infection[59,65] whereas *H pylori* infection seemed not to increase the risk of ATD in individuals with dyspeptic symptoms[65]. Taking these results into account, it was proposed that screening for ATD in patients with a positive urea breath test is not indicated[65]. Other studies have failed to show any correlation between *H pylori* infection and ATD in children[66]. Moreover, the similar prevalence of *H pylori* infection, with or without CagA$^+$ strains, in patients with Hashimoto's thyroiditis and controls argues against a true association between *H pylori* infection and Hashimoto's thyroiditis[59]. To further explore the relationship between ATD and *H pylori* infection more clinical trials are required.

Lymphoid follicles in the gastric mucosa are common in ATD, and *H pylori* infection plays a causative role[67]. When an autoimmune disease such as ATD coexists with *H pylori* infection[68], *H pylori* may be involved in the pathogenesis of extra-gastric MALT lymphomas, such as thyroid MALT lymphoma, as shown by a case report describing a primary thyroid MALT lymphoma which occurred in an *H pylori*+ve patient with gastric cancer and Hashimoto's thyroiditis[60]. In this case, after subtotal gastrectomy, the thyroid lymphoma became smaller transiently and when the patient was treated with *H pylori* eradication therapy, the lymphoma completely disappeared. Nevertheless, *H pylori* organisms were not detected in the thyroid lymphoma tissue by polymerase

chain reaction (PCR), questioning the role of *H pylori* in the development of extra-gastric MALT lymphoma in patients with an autoimmune disease[60]. In addition, one study suggested that patients with an autoimmune disease might not be optimal candidates for *H pylori* eradication, even in the case of an early stage gastric MALT lymphoma, since very few of these patients responded to an *H pylori* eradication therapy[68].

On the other hand, it is important to realize that patients with *H pylori*-related gastritis, atrophic gastritis, or both conditions required increased daily doses of T_4 than controls, suggesting that normal gastric acid secretion is necessary for effective absorption of oral T_4[69]. In addition, development of *H pylori* infection in patients treated with T_4 led to an increased serum level of thyrotropin (TSH), an effect that was nearly reversed after eradication of *H pylori* infection[69].

H pylori AND DYSLIPIDEMIA

H pylori infection may cause dyslipidemia, as it leads to elevated levels of total cholesterol[70,71], low-density lipoprotein cholesterol (LDL-c)[71,72], lipoprotein Lp(a)[71], apolipoprotein apo-B[73], triglyceride concentrations[72,74,75] and decreased levels of high-density lipoprotein cholesterol (HDL-c)[73-78] and apolipoprotein apoA-1 concentration in the blood[73,75]. In addition, plasma levels of cholesterol and LDL-c were significantly higher in *H pylori*+ve patients with ischemic stroke compared to *H pylori*-ve patients[70]. It was postulated that chronic *H pylori* infection may shift lipid profiles towards an atherogenic direction *via* the action of pro-inflammatory cytokines, such as interleukins 1 and 6 (IL-1 and IL-6), interferon-α (INF-α) and tumour necrosis factor-α (TNF-α). These cytokines are capable of affecting lipid metabolism in different ways, including activation of adipose tissue lipoprotein lipase, stimulation of hepatic fatty acid synthesis and influencing lipolysis[71,79]. This atherogenic modified lipid profile created by *H pylori* infection may increase the risk for cardiovascular and cerebrovascular diseases, by participating in the process of atherogenesis, especially when Cag-A^+ cytotoxic strains of *H pylori* are present[80,81], although other studies do not support this hypothesis[71,82,83].

According to other studies, *H pylori* infection did not cause any significant changes in plasma levels of total cholesterol[78,84], triglycerides[78,84], LDL-c[78,84] and Apo-B[78,85].

The relationship between dyslipidemia and *H pylori* eradication is also controversial. After one year of eradication of *H pylori* in patients with duodenal ulcers, a significant increase of HDL-c, apo-AI and apo-AII levels was observed in the study by Scharnagl *et al*[86]. Moreover, eradication of *H pylori* in healthy subjects seems to increase HDL-c and decrease LDL-c levels[78]. Also, 6 mo following successful eradication of *H pylori* infection the plasma levels of total cholesterol and LDL-c were found to be significantly lower than those in *H pylori*+ve controls and *H pylori*+ve patients with stroke[70].

In contrast, one study showed that eradication of *H pylori* is associated with minor lipid changes[84], while others showed a significant increase in the incidence of hyperlipidemia in patients with peptic ulcer disease, as serum total cholesterol and triglycerides were elevated in these patients after eradication of *H pylori*[46,47,87].

Table 2 Endocrine disorders and eradication of *H pylori*

Endocrine disorders	*H pylori* eradication
Autoimmune thyroid diseases	↓ of thyroid auto-antibodies[64] ↓ of thyrotropin in *H pylori*+ve patients treated with T4[69]
Diabetes mellitus	↓ in diabetics more than in non-diabetics[20,30-32] ↓ in type I diabetic patients on standard triple therapy more than non-insulin dependent diabetic subjects, regardless of the dosage and/or the duration of therapy[20,31,32]
Dyslipidemia	↑ of HDL-c, apo-AI and apo-AII levels in patients with duodenal ulcers, after 1 year[86] ↑ of HDL-c and ↓ LDL-c levels in healthy subjects[78] ↓ of total cholesterol and LDL-c after 6 mo in *H pylori*+ve controls and *H pylori*+ve patients with stroke[70] ↔ of lipids in patients submitted for endoscopy[84] ↑ of total cholesterol and triglycerides in patients with peptic ulcer disease[46,47] or without[87]
Obesity	↑ of BMI in patients with peptic ulcer disease[46,47] ↑ of the appetite of asymptomatic patients, due to ↑ of plasma ghrelin[48] and ↓ of leptin levels[49,50] ↔ of plasma ghrelin levels in subjects referred for upper gastrointestinal endoscopy[44,57]

BMI: Body mass index; HDL-c: High-density lipoprotein cholesterol; apo-AI: Apolipoprotein AI; apo-AII: Apolipoprotein AII; LDL-c: Low-density lipoprotein cholesterol; +ve: Positive.

CONCLUSION

Since the discovery of *H pylori*, a variety of studies, essentially epidemiological or therapeutic trials, case reports and others, have evaluated the potential direct or indirect involvement of this bacterium in the pathogenesis of various extra-gastric diseases or disorders, amongst them disorders of the endocrine system. A critical review of data published on these proposed associations suggests a strong link between dyslipidemia and *H pylori* infection, whereas increasing evidence emerges on the role of *H pylori* infection in thyroid autoimmune diseases. On the contrary, the association between *H pylori* infection and obesity, PHPT, DM and osteoporosis remains controversial, as evidence is hindered by the small numbers and methodological problems. Therefore, these associations should be interpreted cautiously. Although some evidence suggests that eradication of *H pylori* may lead to an improvement of many endocrine disorders, such as DM, dyslipidemia and autoimmune thyroid disease, excluding obesity (Table 2), more clinical trials are needed in order to confirm this beneficial effect. In conclusion, the causal association between *H pylori* infection and endocrine disorders is still controversial but worthy of further investigation since these diseases affect many people and have a great impact on human health and health economics[88].

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ORIGINAL ARTICLES

Size does not determine the grade of malignancy of early invasive colorectal cancer

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Abstract

AIM: To clarify the clinicopathological characteristics of small and large early invasive colorectal cancers (EI-CRCs), and to determine whether malignancy grade depends on size.

METHODS: A total of 583 consecutive EI-CRCs treated by endoscopic mucosal resection or surgery at the National Cancer Center Hospital between 1980 and 2004 were enrolled in this study. Lesions were classified into two groups based on size: small (≤ 10 mm) and large (> 10 mm). Clinicopathological features, incidence of lymph node metastasis (LNM) and risk factors for LNM, such as depth of invasion, lymphovascular invasion (LVI) and poorly differentiated adenocarcinoma (PDA) were analyzed in all resected specimens.

RESULTS: There were 120 (21%) small and 463 (79%) large lesions. Histopathological analysis of the small lesion group revealed submucosal deep cancer (sm: ≥ 1000 μm) in 90 (75%) cases, LVI in 26 (22%) cases, and PDA in 12 (10%) cases. Similarly, the large lesion group exhibited submucosal deep cancer in 380 (82%) cases, LVI in 125 (27%) cases, and PDA in 79 (17%) cases. The rate of LNM was 11.2% and 12.1% in the small and large lesion groups, respectively.

CONCLUSION: Small EI-CRC demonstrated the same aggressiveness and malignant potential as large cancer.

Key words: Colorectal cancer; Submucosal invasion; Lymph node metastasis; Endoscopic mucosal resection

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INTRODUCTION

Colorectal cancer (CRC) is the third most important cause of cancer mortality in Japan, and its incidence is gradually increasing. To reduce CRC mortality, early detection and appropriate treatment are required. In general, small lesions are suspected of having a lower malignant potential than large ones, and hence are easy to remove endoscopically. Several authors have reported that the malignant potential of early invasive colorectal cancer (EI-CRC) increases with lesion size[1-3]. Therefore, lesion size is considered to be indicative of the depth of invasion and presence of lymph node metastasis (LNM). In contrast, flat, and in particular depressed lesions, are considered to have a tendency to invade rapidly the submucosal layer, even when small[4-6]. However, clinicopathological features of small EI-CRCs have still

not been studied extensively.

The aim of this retrospective study was to clarify the clinicopathological characteristics of small and large EI-CRCs and their implications for endoscopic treatment.

MATERIALS AND METHODS

Subjects

Five hundred and eighty-three patients (374 male and 209 female) with EI-CRC that had been resected surgically or endoscopically at the National Cancer Center Hospital, between January 1980 and January 2004, were examined retrospectively. In all of these patients, cancer cells invaded through the muscularis mucosa into the submucosal layer but did not extend deeply into the muscularis propria. Eligibility also required the lesions to be macroscopically non-pedunculated (sessile, flat and depressed). Patients with synchronous advanced CRC, multiple EI-CRCs, inflammatory bowel disease, hereditary non-polyposis colorectal cancer and familial adenomatous polyposis were excluded from the study.

Methods

All lesions were classified into two groups according to their endoscopic image size: small (≤ 10 mm) and large (> 10 mm). Furthermore, lesions were classified into three categories (sessile, 0- Ⅰs, Ⅰs+Ⅱa; flat, 0-Ⅱa; and depressed, 0-Ⅱc, Ⅱa+Ⅱc, Ⅰs+Ⅱc) according to the Paris classification[7]. Clinicopathological features, incidence of LNM and risk factors for LNM, such as depth of invasion, lymphovascular invasion (LVI) and poorly differentiated adenocarcinoma (PDA) were analyzed in all resected specimens.

Histopathology

Resected specimens were fixed in 10% formalin and examined histopathologically following hematoxylin and eosin staining. Histopathological diagnosis was based on the World Health Organization (WHO) criteria[8]. Submucosal invasion was measured from the muscularis mucosa to the deepest portion. When the muscularis mucosa could not be identified because of cancer invasion, the vertical length was measured from the surface of the lesion to the deepest portion according to Kitajima's classification[9]. Tumors with a vertical length of < 1000 μm in the submucosal layer were classified as submucosal superficial invasive cancers (sm-superficial), and lesions with a length ≥ 1000 μm were classified as submucosal deep invasive cancers (sm-deep). The tumor growth patterns were histopathologically divided into polypoid growth (PG) and non-polypoid growth (NPG) types. Shimoda *et al*[10] have reported polyp cancers with protrusions caused by intramucosal proliferation of the carcinoma or coexistent adenoma that behaved as PG type carcinoma, while flat/depressed type carcinoma without polypoid proliferation of intramucosal tumor behaved as NPG type carcinoma.

Statistical analysis

The significance of differences in proportions was assessed by the χ^2 test, Fisher's exact test and the Wilcoxon matched-pairs signed-ranks test using SPSS statistical software (SPSS for Windows , version 16.0J, Tokyo, Japan). Statistical significance was defined as $P < 0.05$.

RESULTS

A total of 583 EI-CRCs were retrospectively evaluated, with 120 (21%) small and 463 (79%) large lesions identified (Table 1). The gender ratio (male/female) was 2.4 and 1.7, and the mean age was 61.5 and 62.4 years in the small and large lesion groups, respectively. Mean size of the small and large lesions was 8.3 and 22.1 mm, respectively.

Macroscopic type, growth type and location

Macroscopic assessment of small lesions identified 51 cases as sessile (42%), 14 as flat (12%), and 55 as depressed (46%). Similarly, large lesion groups comprised 233 sessile (50%), 64 flat (14%), and 166 depressed (36%) type. PG types were identified in 32% (38/120) and 54% (250/463) of small and large lesions, respectively. In contrast, the prevalence of NPG type in the small lesion group was significantly higher than in the large lesion group (68% *vs* 46%, $P < 0.0001$). Regarding tumor location, there were 33 (27%) rectal, 56 (47%) distal colon and 31 (26%) proximal colon cancers in the small lesion group. In contrast, there were 213 (46%) rectal, 139 (30%) distal colon and 111 (24%) proximal cancers in the large lesion group. The incidence of rectal cancer in the large lesion group was significantly higher than in the small lesion group ($P = 0.02$).

LNM

Among the lesions treated surgically, the incidence of LNM was 11.2% (10/89) and 12.1% (46/381) in small and large lesion groups, respectively ($P = 0.85$) (Table 2).

Depth of invasion/LVI/PDA

Histopathological analysis of the small lesion group revealed sm-deep cancer in 90 (75%) cases, LVI in 26 (22%) and PDA in 12 (10%). Similarly, the large lesion group exhibited sm-deep cancer in 380 (82%) cases, LVI in 125 (27%), and PDA in 79 (17%). Therefore, in relation to depth of invasion, LVI and PDA, there were no significant differences between the groups.

Treatment strategy

Among the small lesion group, 62 (52%) cases were initially treated with endoscopic mucosal resection (EMR), while 58 (48%) cases were surgically resected. In contrast, among the large lesion group, 133 (29%) cases were initially treated with EMR, while 330 (71%) cases were surgically resected. Among all lesions treated by EMR, there were no differences in the rate of positive and unknown vertical and/or lateral cut margins in the small (18%, 11/62) and large lesion groups (20%, 26/133). Furthermore, among all positive cut margin cases in the small and large lesion groups, there were 11 (100%) and 18 (69%) positive vertical margin cases (Table 3, Figures 1 and 2).

Table 1 Comparison of clinicopathological and endoscopic characteristics for 583 study cases

	Small (≤ 10 mm)	Large (> 10 mm)	*P* value
No. of lesions, *n* (%)	120 (21)	463 (79)	
Gender (M/F)	85/35	289/174	0.09
Age (yr), mean (range)	61.5 (39-84)	62.4 (30-90)	0.86
Macroscopic type, *n* (%)			
Sessile (0-Ⅰs, Ⅰs+Ⅱa)	51 (42)	233 (50)	0.13
Flat (0-Ⅱa)	14 (12)	64 (14)	
Depressed (0-Ⅱc, Ⅱa+Ⅱc, Ⅰs+Ⅱc)	55 (46)	166 (36)	
Size (mm), mean ± SD	8.3 ± 1.6	22.1 ± 9.6	
Growth pattern (PG/NPG)	38/82	250/213	< 0.0001
Location, *n* (%)			
Rectum	33 (27)	213 (46)	0.02
Distal colon[1]	56 (47)	139 (30)	
Proximal colon[2]	31 (26)	111 (24)	

[1]Descending-sigmoid colon; [2]Cecum-transverse colon.

Table 3 Comparison of treatment strategy and positive rate of cut margin *n* (%)

	Small (≤ 10 mm)	Large (> 10 mm)	*P* value
Initial treatment			
EMR	62 (52)	133 (29)	< 0.0001
Surgery	58 (48)	330 (71)	
Positive rate of cut margin[1]	11 (18)	26 (20)	0.81
In EMR cases			
Lateral	0 (0)	8 (31)	0.08
Vertical	11 (100)	18 (69)	

[1]Positive and unknown cut margin. EMR: Endoscopic mucosal resection.

According to the initial treatment, there were 134 (69%) and 336 (87%) sm-deep cancers in the EMR and surgery groups, respectively. Furthermore, there were 33 (17%) and 118 (30%) LVI-positive, and 18 (9%) and 73 (19%) PDA-positive cases in the EMR and surgery groups, respectively. There were 37 (19%) positive cut margin cases, including 29 (78%) positive vertical margins in the EMR group. In contrast, there were no positive cut margin cases in the surgery group. In the EMR group, 82 (42%) patients underwent additional surgery with LN dissection after EMR within 6 mo. The incidence of LNM was 11.0% (9/82) and 12.1% (47/388) in the EMR and surgery groups, respectively ($P = 0.79$) (Table 4).

DISCUSSION

Several authors have reported a strong association between lesion size and submucosal invasion or risk of LNM when referring to the grade of malignancy of early CRC. Large lesion size has been considered an indicator of deep submucosal invasion and presence of LNM. However, in this large retrospective study, small EI-CRC demonstrated a similar aggressive behavior and malignant potential to those of large lesions, with a similar risk of LNM , LVI and PDA among both groups.

Intramucosal CRC is thought generally to have no potential for LNM. In contrast, it has been reported that

Table 2 Incidence of LNM and clinicopathological characteristics based on tumor size *n* (%)

	Small (≤ 10 mm)	Large (> 10 mm)	*P* value
LNM	10/89 (11.2)	46/381 (12.1)	0.85
Depth of invasion			
sm-superficial (< 1000 μm)	30 (25)	83 (18)	0.08
sm-deep (≥ 1000 μm)	90 (75)	380 (82)	
LVI	26 (22)	125 (27)	0.23
PDA	12 (10)	79 (17)	0.06

LVI: Lymphovascular invasion; PDA: Poorly differentiated adenocarcinoma; LNM: Lymph node metastasis.

Table 4 Comparison of clinicopathological characteristics and incidence of LNM based on the treatment strategy *n* (%)

	EMR (*n* = 195)	Surgery (*n* = 388)	*P* value
Depth of invasion			
sm-superficial (< 1000 μm)	61 (32)	52 (13)	< 0.0001
sm-deep (≥ 1000 μm)	134 (69)	336 (87)	
LVI	33 (17)	118 (30)	0.0006
PDA	18 (9)	73 (19)	0.0006
Positive rate of cut margin[1]	37 (19)	0 (0)	< 0.0001
Lateral	8 (22)	0 (0)	
Vertical	29 (78)	0 (0)	
Additional surgical operation	82 (42)	-	
LNM	9/82 (11.0)	47/388 (12.1)	0.79

[1]Positive and unknown cut margin.

LNM occurs in 6%-13% of patients with submucosal invasive CRC[11-15]. Therefore, radical surgery with LN dissection is recommended strongly in these cases. At present, EMR provides an endoscopic cure of early stage CRC when there is no risk of LNM. Advances in endoscopic instruments and techniques have increased the detection rates of early stage CRC and have expanded the indications for EMR[16].

In the past 20 years, many investigators have proposed the following histopathological criteria when considering additional surgery after EMR of submucosal cancers: massive submucosal invasion (≥ 1000 μm), and/or LVI, and/or PDA[17-22]. Among these factors, LVI and PDA are impossible to predict before resection. At this point, it is crucial to predict the vertical depth of invasion of submucosal cancers prior to EMR. In our center, we use routinely a magnifying colonoscope to decide on the adequate treatment of early stage CRC. Magnifying chromoendoscopy (MCE) is a standardized validated method that facilitates detailed analysis of the morphological architecture of colonic mucosal crypt orifices (pit pattern), in a simple and rapid manner. We have reported previously the efficacy of MCE to diagnose an invasive pattern as a typical finding of sm-deep cancers, and have demonstrated that it provides a good correlation between pit pattern and tumor depth in flat and depressed CRC[23-27].

Many authors have reported that depressed and/or NPG type lesions are considered to have a high malignant potential, compared to the polypoid type lesions of similar

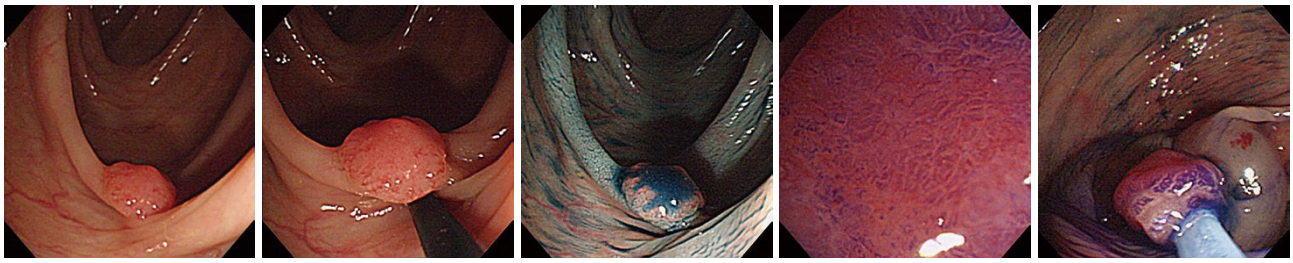

Figure 1 The lesion was located in the transverse colon. Endoscopic examination revealed a flat, elevated lesion with a central depression, which was macroscopically diagnosed as 0-Ⅱa+Ⅱc. The high-magnification view revealed a typical type Ⅵ pit (invasive) pattern on the depressed margin. The final endoscopic diagnosis was a 0-Ⅱa+Ⅱc type early colon cancer with submucosal deep invasion. However, patient strongly hoped EMR as an initial treatment. We performed EMR after injecting normal saline into the submucosa.

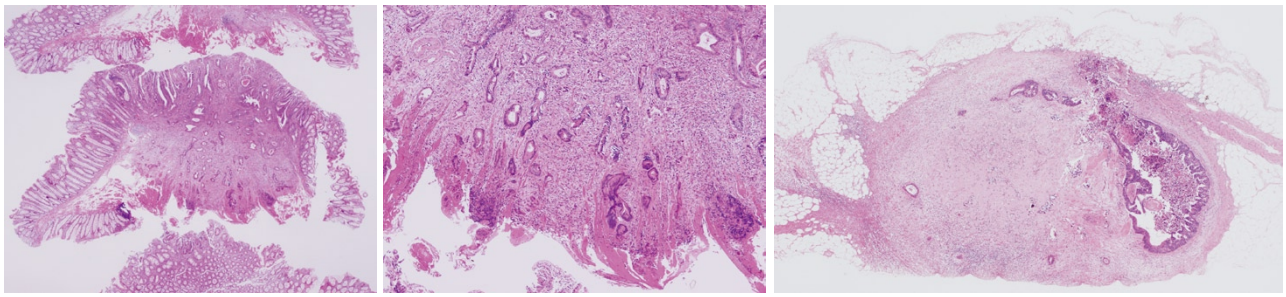

Figure 2 The final histopathological diagnosis was early invasive colon cancer, well-differentiated adenocarcinoma, sm-deep, NPG type, ly (-), v (-), cut end (+) (vertical margin positive). Since cancer was exposed in the vertical cut margin, additional surgical resection was performed and LNM was detected.

size[4,28-31]. Kurisu *et al*[20] have investigated the development and progression of EI-CRC. In that study, NPG lesions were significantly smaller in size (14.2 mm *vs* 24.2 mm) but showed deeper infiltration than PG types. They concluded that tumor development and the degree of invasion differed significantly between the two types of carcinoma. On the other hand, non-polypoid colorectal neoplasms (NP-CRNs) have been reported recently in the United States. Soetikno *et al*[31] have reported the prevalence of NP-CRNs in a veterans' hospital population. The overall prevalence of NP-CRNs and NP-CRNs with in situ or submucosal invasive carcinoma was 9.35% and 0.82%, respectively. They also concluded that NP-CRNs were more likely to contain carcinoma (OR: 9.78) than polypoid lesions, regardless of size. In the present study, small EI-CRCs ≤ 10 mm in diameter showed a significantly higher incidence of NPG type lesions than in the large lesion group ($P < 0.0001$). However, there was no significant difference in proportion of the macroscopic type between the groups ($P = 0.13$). Among the lesions diagnosed as Is type (sessile) in the small lesions group, 47% (14/30) were classified as NPG type histopathologically. From these results, we conclude that further investigation is required to confirm the growth pattern, especially for small sessile lesions diagnosed during colonoscopy.

In contrast, the rate of EMR as an initial treatment was 33% (195/583) in our study. In particular, it was significantly higher in the small lesion than the large lesion group (52% *vs* 29%, $P < 0.0001$). Among the 195 lesions removed by EMR as an initial treatment in both groups, 61 cases (32%) were sm-superficial cancers. On the other hand, there was no significant difference in the positive rate of cut margins between the small and large lesion groups (18% *vs* 20%). This result implies that EMR should not be performed readily for EI-CRC, from the viewpoint of no-touch isolation[32] and EMR complications. Intramucosal lesions (adenoma or intramucosal cancer) are usually well lifted by submucosal injection. In contrast, invasive cancer, especially sm-deep cancer, cannot be lifted because of the presence of submucosal fibrosis or desmoplastic reaction. Uno *et al*[33] have reported this phenomenon as the "non-lifting sign". Kobayashi *et al*[34] have reported, among 271 colorectal neoplastic lesions, that the non-lifting sign of deeper infiltration had a sensitivity of 61.5%, specificity of 98.4%, and accuracy of 94.8%. In contrast, endoscopic diagnosis had a sensitivity of 84.6%, specificity of 98.8%, and accuracy of 97.4%, with statistically significant differences in terms of sensitivity and accuracy. Furthermore, since submucosal injection varies depending on the expertise of the endoscopist, we consider that an endoscopic diagnosis is much more important and accurate when endoscopic resection is considered as the therapeutic option.

There are some limitations to this study. Firstly, this was a single-center study, and although the number of examined EI-CRCs was adequate, a multicenter analysis should be performed to clarify the clinical importance of small EI-CRCs. In addition, this study was carried out retrospectively between 1980 and 2004. In relation to endoscopic treatment for early CRC, endoscopic submucosal dissection (ESD) technique and Glycerol/Sodium hyaluronate as an injected solution during EMR has made progress recently[35,36]. In particular, ESD provides not only an *en bloc* large specimen but also

negative lateral and vertical cut margins.

In conclusion, with regard to the risk of LNM, small EI-CRCs demonstrate the same aggressiveness and malignant potential as large lesions. Moreover, from the perspective of the concept of no-touch isolation, therapeutic cost, and complications during EMR, special attention must be paid when treating even small early stage lesions, especially NPG type lesions.

COMMENTS

Background

In general, small colorectal lesions are suspected of having a lower malignant potential than large ones, and hence are easy to remove endoscopically. Several authors have reported that the malignant potential of early invasive colorectal cancer (EI-CRC) increases with lesion size.

Research frontiers

The aim of this retrospective study was to clarify the clinicopathological characteristics of small (≤ 10 mm) and large (> 10 mm) EI-CRCs.

Innovations and breakthroughs

A total of 583 EI-CRCs were evaluated retrospectively, with 120 (21%) small and 463 (79%) large lesions identified. With regard to the risk of lymph-node metastasis (LNM), small EI-CRCs demonstrate the same aggressiveness and malignant potential as large lesions.

Peer review

The authors examined retrospectively a large group of patients with EI-CRCs gathered over 20 years in a national cancer hospital, and demonstrated that small EI-CRCs (≤ 10 mm) had the same aggressiveness and malignant potential as large cancers. Special attention must be paid when treating even small lesions.

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ORIGINAL ARTICLES

Higher CO_2-insufflation pressure inhibits the expression of adhesion molecules and the invasion potential of colon cancer cells

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Abstract

AIM: To investigate the influence of CO_2-insufflation pressure on adhesion, invasion and metastatic potential of colon cancer cells based on adhesion molecules expression.

METHODS: With an *in vitro* artificial pneumoperitoneum model, SW1116 human colon carcinoma cells were exposed to CO_2-insufflation in 5 different pressure groups: 6 mmHg, 9 mmHg, 12 mmHg, 15 mmHg and control group, respectively for 1 h. Expression of E-cadherin, ICAM-1, CD44 and E-selectin was measured at 0, 12, 24, 48 and 72 h after CO_2-insufflation using flow cytometry. The adhesion and invasion capacity of SW1116 cells before and after exposure to CO_2-insufflation was detected by cell adhesion/invasion assay *in vitro*. Each group of cells was injected intraperitoneally into 16 BALB/C mice. The number of visible abdominal cavity tumor nodules, visceral metastases and survival of the mice were recorded in each group.

RESULTS: The expression of E-cadherin, ICAM-1, CD44 and E-selectin in SW1116 cells were changed significantly following exposure to CO_2 insufflation at different pressures ($P < 0.05$). The expression of E-cadherin, CD44 and ICAM-1 decreased with increasing CO_2-insufflation pressure. The adhesive/invasive cells also decreased gradually with increasing pressure as determined by the adhesion/invasion assay. In animal experiments, the number of abdominal cavity tumor nodules in the 15 mmHg group was also significantly lower than that in the 6 mmHg group (29.7 ± 9.91 *vs* 41.7 ± 14.90, $P = 0.046$). However, the survival in each group was not statistically different.

CONCLUSION: CO_2-insufflation induced a temporary change in the adhesion and invasion capacity of cancer cells *in vitro*. Higher CO_2-insufflation pressure inhibited adhesion, invasion and metastatic potential *in vitro* and *in vivo*, which was associated with reduced expression of adhesion molecules.

Key words: Adhesion molecule; Colorectal cancer; Metastasis; Pneumoperitoneum; Artificial; Tumor invasion

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Ma JJ, Feng B, Zhang Y, Li JW, Lu AG, Wang ML, Peng YF, Hu WG, Yue F, Zheng MH. Higher CO_2-insufflation pressure inhibits the expression of adhesion molecules and the invasion potential of colon cancer cells. *World J Gastroenterol* 2009; 15(22): 2714-2722 Available from: URL: http://www.wjgnet.com/1007-9327/15/2714.asp DOI: http://dx.doi.org/10.3748/wjg.15.2714

INTRODUCTION

The laparoscopic approach is generally considered

to be less invasive and less immunosuppressive than conventional open surgery[1]. However, port site metastases and abdominal wall recurrences although rare nowadays, remain a problem with laparoscopic surgery for cancer[2]. Recently, several studies demonstrated that the five-year survival rates were similar after laparoscopically assisted colectomy and open colectomy for cancer patients, suggesting that the laparoscopic approach is an acceptable alternative to open surgery for colorectal cancer[3-5]. However, whether CO_2 insufflation, which is widely used in laparoscopic surgery, increases the metastatic potential of tumor cells is still the focus of related studies[6-8].

Adhesion molecules play an important role in cell-cell, cell-extracellular matrix (ECM) interactions at tumor metastasis[9,10]. ICAM-1, E-cadherin, and CD44 are representative molecules involved in the interaction not only between the adhesion of inflammatory cells but also between tumor cells and the endothelium. Previous studies have shown that reduced E-cadherin expression correlates with increased invasiveness in colorectal carcinoma cell lines[11]. The decrease or deletion of E-cadherin was reported to induce liver metastasis of colorectal tumors[12,13], and the increased expression of CD44 also induces tumor metastasis[14]. Recently it has been reported that CO_2 insufflation can effect the expression of adhesion molecules, such as E-cadherin, CD44 and ICAM-1, which are related to tumor metastasis[15-17]. However, there are few studies on the influence of CO_2-insufflation pressure on adhesion molecules and how CO_2 insufflation further impacts on metastatic potential following altered expression of these adhesion molecules.

In this study, we investigated whether expression of adhesion molecules are changed after CO_2 insufflation *in vitro*. We further investigated the influence of CO_2 pressure on the expression of these adhesion molecules, and how CO_2-insufflation influenced the adhesion and invasion potential of colon cancer cells *in vitro* and *in vivo*.

MATERIALS AND METHODS

Cell culture

The human colorectal adenocarcinoma cell line SW1116 (human, Caucasian, colon, adenocarcinoma, grade Ⅲ, CCL-233™) was cultured in RPMI 1640 medium (Hangzhou Genom Co. Cat. No. GNM31800.25) supplemented with 10% fetal calf serum (Life Technologies). The human colorectal adenocarcinoma cell line Lovo (human, colon, adenocarcinoma, grade IV, CCL-229™) used in the invasive assay was cultured in F12K medium (Hangzhou Genom Co. Cat. No. GNM21700.25) supplemented with 10% fetal calf serum. The monolayer cell culture was maintained in culture flasks under standard culture conditions at 37℃ in air with 5% CO_2.

Monoclonal antibodies

For flow cytometry, the following monoclonal antibodies (Mab) were used: anti-ICAM-1-PE-Cy5 (Clone HA58) and anti-E-selectin-PE (Clone 68-5H11). They were purchased from BD Biosciences (San Diego, CA, USA). Anti-CD44-FITC (Clone J.173) and unconjugated anti-E-cadherin (Clone 67A4) were purchased from Beckman Coulter™. Secondary goat anti-mouse IgG-PE, isotype control mouse IgG-PE-Cy5, and mouse IgG-PE were purchased from BD Biosciences. Mouse IgG FITC, and mouse IgG PE were purchased from Beckman Coulter™.

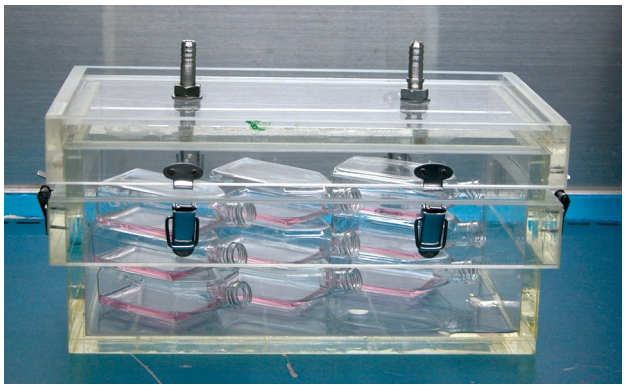

Figure 1 Five litres volume airtight Perspex box with two ventilating ports for pneumoperitoneum establishment *in vitro*.

Animals

Four to eight week-old male BALB/C mice (Chinese Academy of Sciences, Shanghai, China) were kept under standard laboratory conditions and fed a standard laboratory diet with access to water added *libitum* before and after injection.

In vitro pneumoperitoneum establishment

An *in vitro* pneumoperitoneum was established by connecting an electronic CO_2 insufflator (Stryker® Endoscopy) to a 5 L volume airtight Perspex box with two ventilating pits (Figure 1). Medical grade carbon dioxide at a concentration of 99.5% was used. To ensure exhaustion of air in the box, 10 L CO_2 were insufflated into the Perspex box before the tumor cells were placed into the box. The cells were divided into 5 groups with different insufflation pressures (6 mmHg, 9 mmHg, 12 mmHg, 15 mmHg, and room air as a control). The tumor cells and the boxes were transferred into an incubator at 37℃ and the *in vitro* pneumoperitoneum model was retained for 1 h at different insufflation pressures during a continuous CO_2 flow of 2.5 L/min.

Flow cytometry (FACScan)

At 0, 12, 24, 48, and 72 h after exposure, the cells were detached from the tissue culture flasks using ice-cold phosphate-buffered saline (PBS) containing 0.1% sodium azide and resuspended at 5×10^5 cells/mL. One milliliter of the cell suspension was incubated with the monoclonal antibodies (1:100). The cells (5×10^5/group) were incubated with anti-ICAM-1-PE-Cy5, 10 μL, anti-E-selectin-PE, 10 μL, anti-CD44-FITC, 20 μL and unconjugated anti-E-cadherin, 10 μL for 30 min at room

temperature. At the end of the incubation period, the cells were washed with PBS. After treatment with unconjugated anti-E-cadherin the cells were incubated with a secondary goat anti-mouse IgG-PE (3 μL/5 × 10^5 cells) for 30 min at room temperature. After washing with PBS, the cells were fixed with 0.5% formalin, and analyzed for surface receptor expression by flow cytometry (BD Calibur). The expression of 4 different cell adhesion molecules was measured at 5 time points (0, 12, 24, 48 and 72 h) after CO_2 insufflation. Expression of these adhesion molecules is given as mean fluorescence intensity of the cell population.

Adhesion assay

A cell adhesion assay (Cat. No. ECM 554, Chemicon International, USA) was used, in which the wells were coated with human fibronectin, one of the component proteins of the extracellular matrix (ECM). At 0 h and 72 h after exposure to CO_2 insufflation (at 6 mmHg, 9 mmHg, 12 mmHg, 15 mmHg and room air for 1 h, respectively), the cells were detached using 0.25% trypsin and washed with PBS. The wells were rehydrated with 200 μL of PBS per well for at least 15 min at room temperature before being plated at a concentration of 1 × 10^5 cells per well. After the plate was incubated at 37℃ in air with 5% CO_2 for 1 h, each well was washed gently 3 times with PBS. The cells which had adhered to the wells were then dyed with crystal violet and incubated for 5 min at room temperature. After the plate was gently washed 3 times with PBS, 100 μL of Solubilization Buffer (a 50/50 mixture of 0.1 mol/L NaH_2PO_4, pH 4.5 and 50% ethanol) was added to each well. The plate was shaken at room temperature gently to solubilize the cell-bound stain completely. Finally, a microplate reader was used to determine the absorbance at 570 nm as a measure of tumor cell adhesion on the ECM layer.

Cell invasive assay

The SW1116 cells were starved by incubation in serum-free RPMI 1640 for 18-24 h prior to assay. At 0 h and 72 h after CO_2 insufflation (6 mmHg, 9 mmHg, 12 mmHg, 15 mmHg and room air for 1 h, respectively), the cells were harvested by trypsinization, and resuspended in RPMI 1640 at 1 × 10^6 cells/mL. Twenty-four-well QCM™ Cell The Invasion Assay kit (Cat. No. ECM 101, Chemicon International, USA) was used. After rehydration of the ECM layer by adding 300 μL of pre-warmed serum-free medium to the interior of the inserts for 30 min at room temperature, 250 μL of the medium was removed from the inserts without disturbing the membrane. The prepared cell suspension (250 μL) as described previously was then added into each insert, and RPMI 1640 with 5% BSA was added to the lower chamber. After incubation for 24 h at 37℃ in the incubator with 5% CO_2, the medium and suspended cells were removed and the invasion chamber was placed into a clean well containing 225 μL of the cell detachment solution. The cells were then incubated at 37℃ for 30 min, and both lyses buffer and dye solution were added to each well containing the cell detachment solution with cells which had invaded the ECM coated membrane. Finally, the mixture was detected by a fluorescence plate reader using a 480/520 nm filter set. The fluorescence intensity was measured as an index of the quantity of invasive cells. Lovo cells underwent the same procedure in the invasive assay.

Induction of intra-abdominal tumors

To analyze the potential for abdominal cavity dissemination in each group of cells (exposed to CO_2 insufflation at 6 mmHg, 9 mmHg, 12 mmHg, 15 mmHg and room air), each group of cells was injected intraperitoneally into 16 BALB/C mice (1 × 10^6 cells/mouse). Fourteen days later, 10 mice in each group were sacrificed by cervical dislocation, and the number of visible abdominal cavity tumor nodules, reflecting the metastatic potential, was counted. The rate of viscera (liver, kidney, spleen, peritoneum or mesentery) metastasis was also recorded in each group. The remaining mice in each group were monitored for their survival.

Statistical analysis

For studies on the expression of adhesion molecules, each experiment was performed in triplicate. Cell adhesion and invasion assays were repeated five times. Data are expressed as mean ± SD. The repeated-measures ANOVA test, one-way ANOVA test and paired *t* test were used to compare the control mean with the various treatment means: adhesion molecules, cell adhesion and invasion assay *in vitro*, and abdominal cavity tumor metastasis *in vivo*. The rates of organ (liver, kidney, spleen, peritoneum or mesentery) metastasis were analyzed using Fisher's exact test. Statistical significance was set at the 5% level.

RESULTS

Effect of CO_2 insufflation on expression of adhesion molecules

Immediately after the 6 mmHg CO_2 insufflation, ICAM-1 (Figure 2A-C) and CD44 expression significantly increased compared to room air controls (F = 106.38, 297.73; $P < 0.01$), while the expression of ICAM-1 decreased at 48 h and 72 h after the 6 mmHg CO_2 insufflation (F = 17222.3, 385.61; $P < 0.01$) (Figure 2A-C). Immediately after the 9 mmHg CO_2 insufflation, E-selectin expression significantly increased compared to the controls (F = 147.75, P = 0.01), and CD44 expression increased significantly compared to controls (F = 39.20, P = 0.025); 24 h after 9 mmHg CO_2 insufflation, E-cadherin expression increased significantly compared to controls (F = 26.79, P = 0.035) (Figure 2D-F), while a significant reduction in E-selectin expression was observed (F = 33.43, P = 0.029). At 48 h after 9 mmHg CO_2 insufflation, a significant reduction in ICAM-1 expression was demonstrated (F = 282.94, $P < 0.01$). At 48 h after 12 mmHg CO_2 insufflation, CD44 expression increased significantly (F = 93.70, P = 0.011), while 72 h after 12 mmHg CO_2 insufflation, the expression of CD44

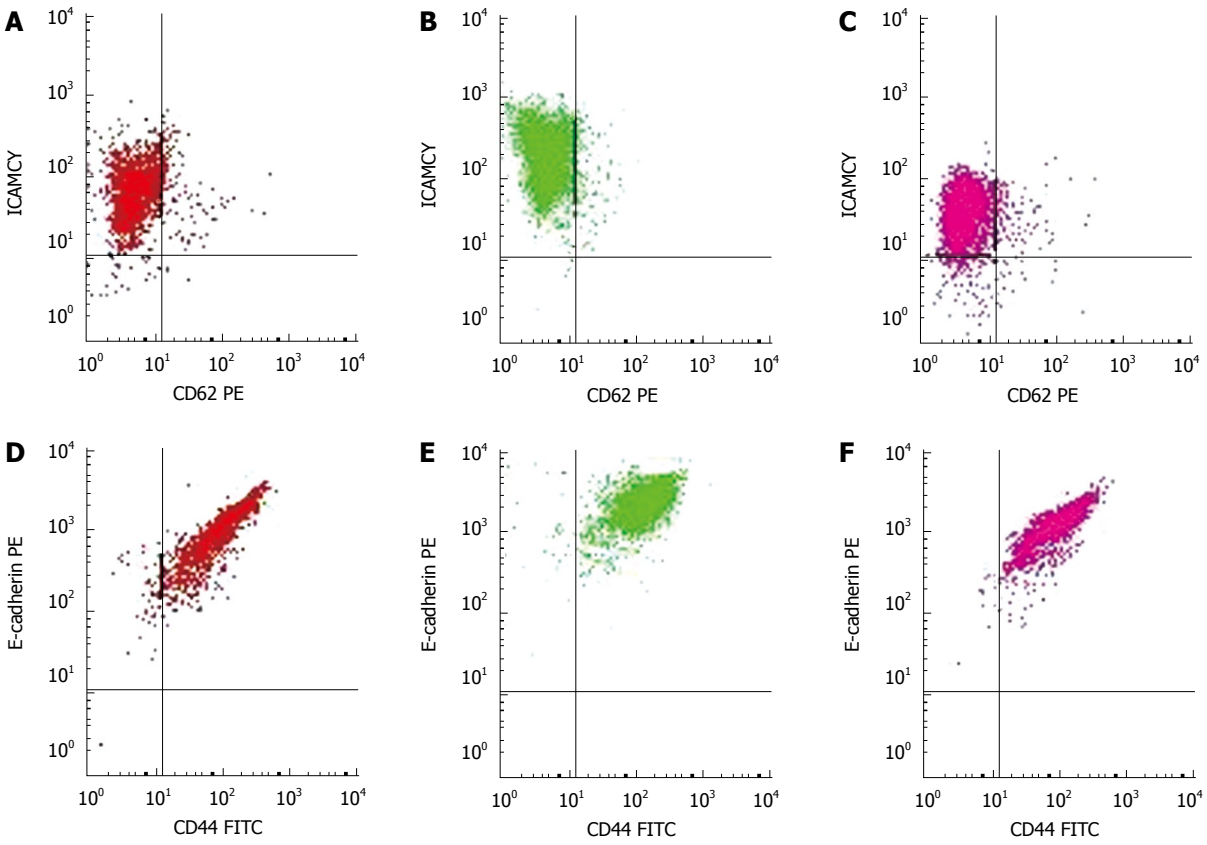

Figure 2 Flow cytometry analysis of ICAM-1 and E-cadherin expression in SW1116. A: ICAM-1 (Room air control); B: ICAM-1 (0 h after the 6 mmHg insufflation); C: ICAM-1 (72 h after the 6 mmHg insufflation); D: E-cadherin (Room air control); E: E-cadherin (24 h after the 9 mmHg insufflation); F: E-cadherin (72 h after the 9 mmHg insufflation). Immediately after the 6 mmHg CO_2 insufflation (B), ICAM-1 expression increased significantly compared to room air controls (A) (P = 0.009), while it was significantly less at 72 h after the 6 mmHg insufflation (C) than control group. Twenty four hours after 9 mmHg CO_2 insufflation (E), E-cadherin expression increased significantly compared to controls (D) (P = 0.035), but returned to the control value at 72 h after the 9 mmHg insufflation (F).

decreased significantly compared to the controls (F = 679.38, P < 0.01). Immediately after the 15 mmHg CO_2 insufflation, CD44 expression decreased significantly compared to the controls (F = 70.01, P = 0.014). At 12 h and 24 h after 15 mmHg CO_2 insufflation, an increased expression of E-cadherin was demonstrated (F = 53.53, 68.14, P = 0.018, 0.014). No significant differences in the expression of these adhesion molecules at other time points and pressures were observed.

Effect of rising insufflation pressure on expression of adhesion molecules

The expression of CD44, E-cadherin and ICAM-1 decreased with increasing CO_2-insufflation pressure. At the 0 h time point, the differences in CD44 expression between the 6 mmHg and the 9 mmHg group, the 9 mmHg and the 12 mmHg group, the 12 mmHg and the 15 mmHg group were significant (t = 4.4291, 4.5725, 7.3587, P < 0.01) (Figure 3A). At the same time point, E-cadherin expression in the 9 mmHg group was less than that in the 6 mmHg group (t = 7.1839, P < 0.01), while in the 12 mmHg and the 15 mmHg group, E-cadherin expression was significantly less than that in the 9 mmHg group (t = 4.5148, 4.4582, P < 0.01) (Figure 3B). ICAM-1 expression in the 9 mmHg, 12 mmHg and 15 mmHg groups was less than that in the 6 mmHg group, respectively (t = 3.3359, 2.3189, 2.7546, P < 0.01, P = 0.046, 0.022), and the expression at 15 mmHg was significantly less than that at 12 mmHg (t = 2.2912, P = 0.048) (Figure 3C).

Effect of CO_2 insufflation on adhesion potential of SW1116 in vitro

Immediately after exposure to increasing CO_2-insufflation pressure, cell adhesion decreased gradually. Cell adhesion in the 15 mmHg group was significantly less than that at 12 mmHg, 9 mmHg, 6 mmHg and the control (all P < 0.01), and cell adhesion in the 12 mmHg group was also less than that in the 9 mmHg, 6 mmHg and control groups (all P < 0.01). The cells exposed to 6 mmHg had more adhesion capacity than those exposed to 9 mmHg or the control group, however, the differences were not significant (P = 0.886, P = 0.058) (Figure 4A). At 72 h after CO_2 insufflation, the differences between the various insufflation pressure groups were not significant (Figure 4B).

Effect of CO2 insufflation on invasion potential of SW1116 and Lovo in vitro

Immediately after exposure to 15 mmHg CO_2 insufflation, the invasion of SW1116 cells decreased significantly compared to the cells before exposure (11.36 ± 0.861 *vs* 13.43 ± 1.113; P = 0.019), while immediately after exposure to 6 mmHg insufflation, invasive cells increased compared to

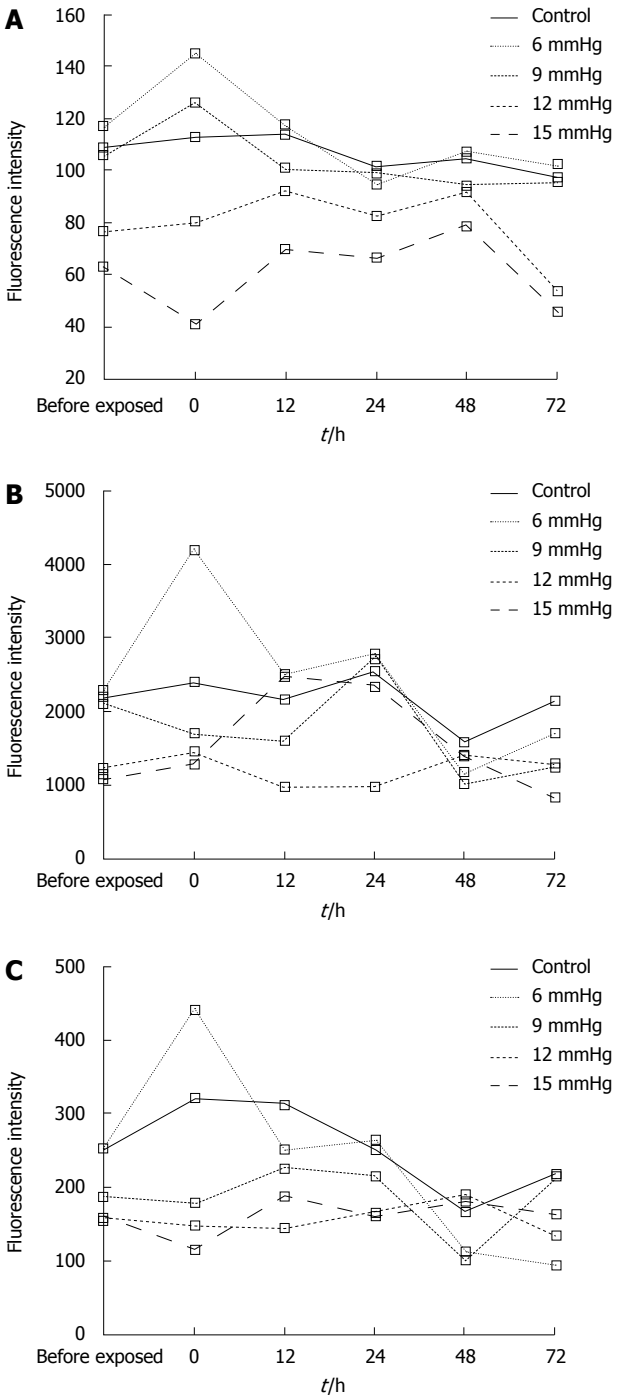

Figure 3 The effect of CO_2 insufflation pressure on expression of adhesion molecules. A: Expression of CD44; B: Expression of E-cadherin; C: Expression of ICAM-1. With increasing CO_2-insufflation pressure, the expression of CD44 (A), E-cadherin (B), and ICAM-1 (C) decreased at some time points, especially at the start (0 h) after CO_2-insufflation.

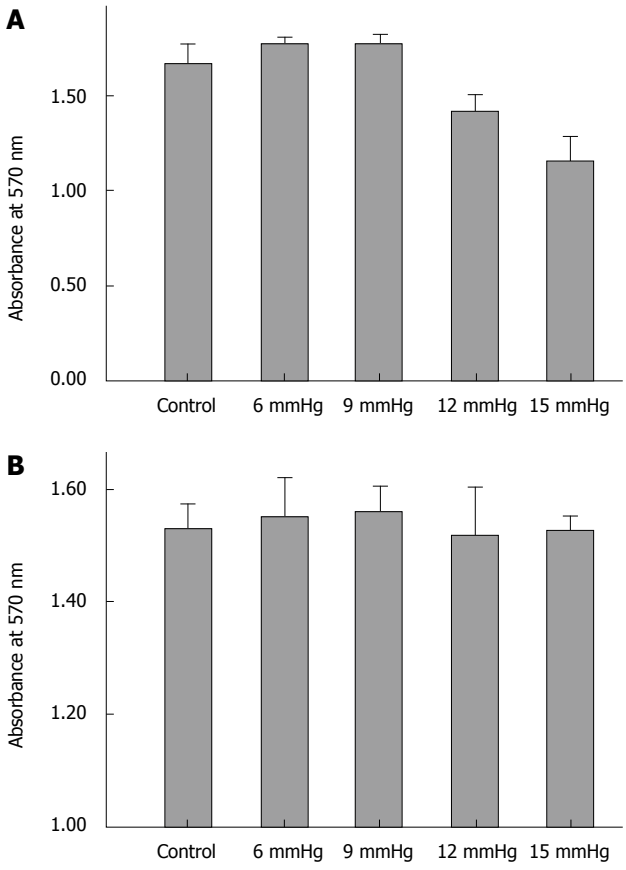

Figure 4 Results of adhesion assay. A: 0 h after CO_2 insufflation; B: 72 h after CO_2 insufflation. At 0 h after CO_2 insufflation (A), with increasing CO_2-insufflation pressure, the adhesion of the SW1116 cell line decreased gradually (12 mmHg *vs* control, $P < 0.001$; 15 mmHg *vs* 12 mmHg, $P < 0.001$). At 72 h after CO_2 insufflation (B), cell adhesion was similar among the different groups ($P > 0.05$).

before exposure (15.58 ± 1.015 *vs* 14.42 ± 1.491; $P = 0.056$), however, the difference was not significant (Figure 5A). At 72 h after CO_2 insufflation, cell invasion was similar to that for cells before exposure (Figure 5B).

The differences between various insufflation pressures were also compared. At the 0 h time point, the cells exposed to 15 mmHg were significantly less invasive than those exposed to the other insufflation pressures (*vs* 6 mmHg, $P < 0.01$; *vs* 9 mmHg, $P = 0.011$; *vs* 12 mmHg, $P = 0.016$; and *vs* room air, $P = 0.014$), and the cells exposed to 6 mmHg were more invasive than cells exposed to the other insufflation pressures (*vs* 9 mmHg, $P = 0.013$; *vs* 12 mmHg, $P < 0.01$; *vs* 15 mmHg $P < 0.01$; and *vs* room air, $P = 0.011$). However, these differences were not significant (Figure 5A). At 72 h after CO_2 insufflation, the differences between the various insufflation pressure groups were not significant (Figure 5B).

The human colon cancer cell line Lovo was also tested in the invasive assay, and a similar trend was observed (Figure 5C and D).

Effect of CO_2 insufflation on metastasis potential of SW1116 in vivo

The ratios of the number of mice which developed intra-abdominal tumors/the number of mice which received inoculation of SW1116 cells in the various groups were as follows: (1) control group, 10/10; (2) 6 mmHg group, 9/10; (3) 9 mmHg group, 9/10; (4) 12 mmHg group, 9/10; (5) 15 mmHg group, 10/10. The differences were not significant. The mean number of intra-abdominal tumor nodules in the various groups was as follows: (1) control group = 36.8 ± 15.32, (2) 6 mmHg group = 41.7 ± 14.90, (3) 9 mmHg group = 33.9 ± 10.29, (4) 12 mmHg group = 33.2 ± 11.72, (5) 15 mmHg = 29.7 ± 9.91. The difference between the 6 mmHg group and the 15 mmHg group was significant ($P = 0.046$) (Figure 6A-C), but the differences among

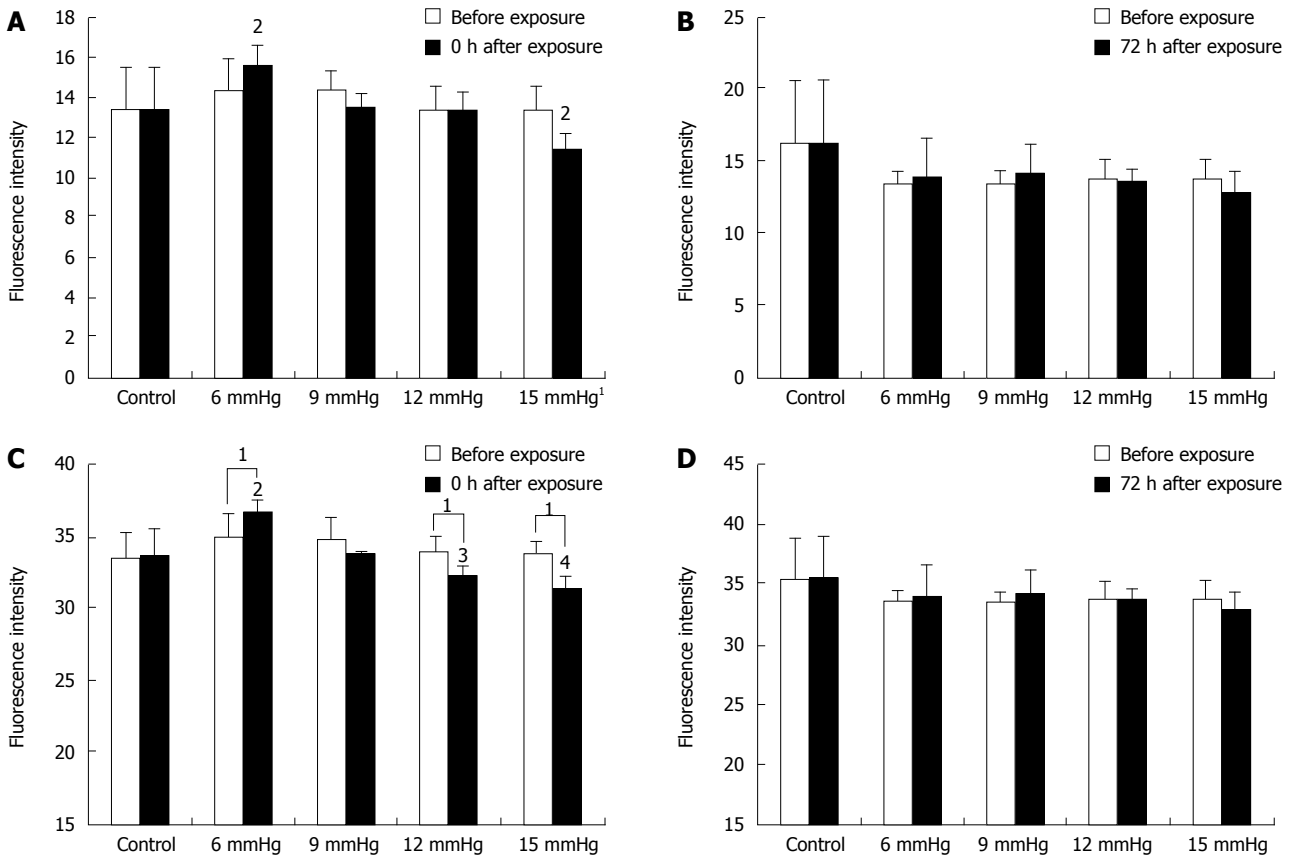

Figure 5 Results of invasion assay. A: SW1116, 0 h after exposure; B: SW1116, 72 h after exposure; C: Lovo, 0 h after exposure; D: Lovo, 72 h after exposure. A: [1]At 0 h after exposure to 15 mmHg CO_2 insufflation, invasive cells decreased significantly compared to the cells prior to exposure (P = 0.019); [2]At 0 h after exposure, invasion of the 6 mmHg or the 15 mmHg group was significantly different from the other pressure groups (P < 0.05). B: The invasion fluorescence intensities of SW1116 before and 72 h after exposure to CO_2 insufflation were not significantly different (P > 0.05). Seventy two hours after the CO_2 insufflation, there were no significant differences between the various insufflation pressure groups (P > 0.05); C: [1]At 0 h after exposure to 15 mmHg, 12 mmHg or 6 mmHg CO_2 insufflation, invasive cells of Lovo decreased significantly compared to the cells prior to exposure (P = 0.008, P = 0.0045, P = 0.043); [2]At 0 h after exposure, cell invasion at 6 mmHg was significantly different from the other pressure groups (P < 0.01); [3]At 0 h after exposure, cell invasion in the 12 mmHg group was significantly less than the 9 mmHg group (P < 0.05); [4]At 0 h after exposure, cell invasion in the 15 mmHg group was significantly less than the 6 and 9 mmHg groups (P < 0.01); D: The invasion fluorescence intensities of Lovo before and 72 h after exposure to CO_2 insufflation were not significantly different (P > 0.05). Seventy two hours after CO_2 insufflation, there were no significant differences between the various insufflation pressure groups (P > 0.05).

the other groups were not. The results of visceral (liver, kidney, spleen, peritoneum or mesentery) metastases (the number of mice with certain visceral metastasis/the number of mice with abdominal cavity metastasis after inoculation of SW1116 cells) are shown in Table 1. No significant differences in survival rate between the various groups were observed (P = 0.426). The overall survival curve is shown in Figure 7.

DISCUSSION

The use of minimal access techniques in the surgical management of colorectal cancer should be safe and feasible in terms of the oncologic effect on the tumor, as compared with conventional surgery. Recent clinical trials in Europe and America have demonstrated that the recurrence and 5-year survival of laparoscopic colorectal cancer surgery are identical to those obtained by open surgery[3-4]. Similar results have also been reported from Hong Kong[18]. However, the occurrence of port-site metastasis and peritoneal metastasis reported in early studies of laparoscopic surgery for cancer led to many studies investigating the biologic effects of positive pressure pneumoperitoneum on tumor growth and the development of trocar recurrences. Several experimental studies suggested that laparoscopic surgery can promote tumor growth[6,19]. Some animal studies have also shown that CO_2, especially at high insufflation pressures increased the incidence of distant metastases[20,21]. However, other researchers failed to confirm these experimental observations in similar animal models[22]. Thus, although the problem of port-site deposits in patients undergoing laparoscopic surgery for cancer has largely been resolved by improved oncological operative techniques, from a biological standpoint, the adverse effect of a sustained positive pressure CO_2 pneumoperitoneum is still debatable, and it is prudent to investigate the mechanism of tumor metastasis induced by CO_2 pneumoperitoneum.

Some adhesion molecules in cancer cells have been reported to play an important role in tumor metastasis. There is evidence that decreased expression of E-cadherin can initiate tumor invasion and metastasis because E-cadherin prevents detachment of tumor cells from

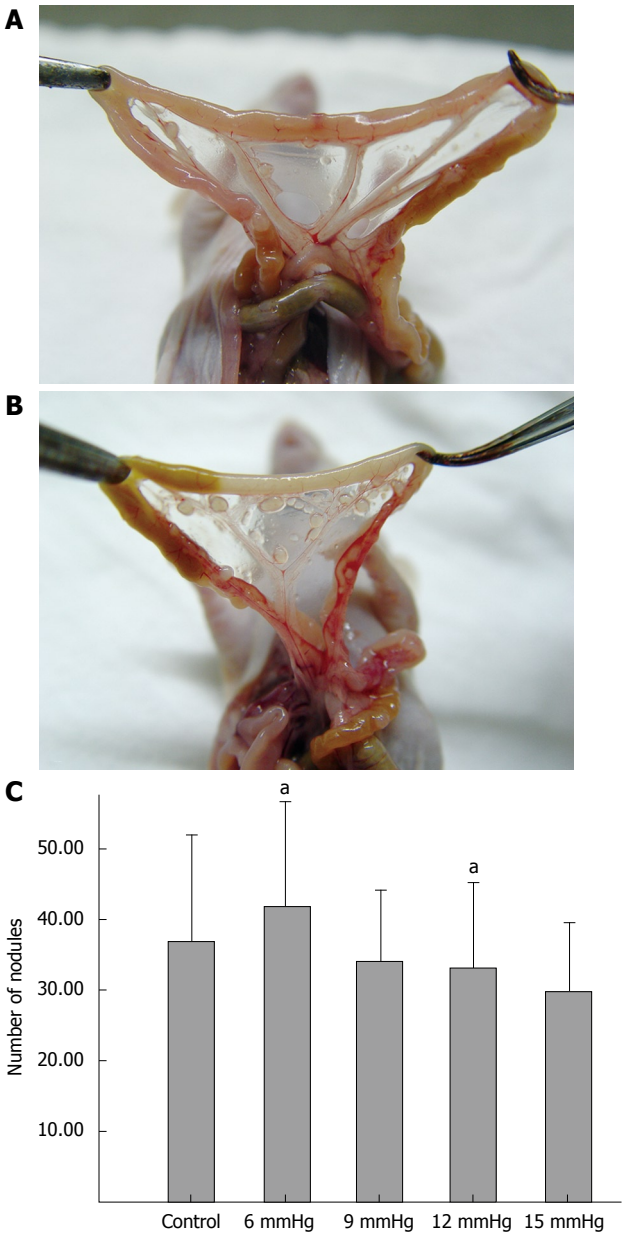

Figure 6 Results of intra-abdominal tumor nodules. A: Mesentery tumor nodules in the 15 mmHg group; B: Mesentery tumor nodules in the 6 mmHg group; C: Numbers of intra-abdominal tumor nodules in the various groups. The number of intra-abdominal tumor nodules in the 15 mmHg group (A) was significantly lower than those in 6 mmHg group (B) ($^aP = 0.046$). The mean numbers of abdominal cavity nodules formed by the cells in each group are described in (C).

the primary tumor[11,23,24]. ICAM-1 mediates the adhesion of circulating cells to the intravascular endothelium. ICAM-1 may also play a role in tumor cell adhesion to the mesothelium[25]. Changes in the expression of CD44 are reported to be related to increased tumor metastasis. The expression of E-selectin in primary colorectal cancer is significantly less than in secondary deposits from this tumor[26]. A number of recent studies have addressed the effects of the laparoscopic environment on these adhesion molecules in cancer cells. Kim *et al*[16] demonstrated a significant alteration in E-cadherin, ICAM-1 and CD44 expression in colon cancer cells after exposure to CO_2. Tahara *et al*[27] reported that CO_2 insufflation increased

Table 1 Distribution of visceral metastases (liver, kidney, spleen, peritoneum or mesentery)

	Liver (n1/n2)	Spleen (n1/n2)	Mesentery (n1/n2)	Kidney (n1/n2)	Peritoneum (n1/n2)
Control	10/10	4/10	10/10	5/10	4/10
6 mmHg	9/9	0/9	9/9	0/9	2/9
9 mmHg	9/9	1/9	9/9	2/9	1/9
12 mmHg	8/9	1/9	9/9	4/9	0/9
15 mmHg	10/10	1/10	10/10	4/10	1/10
P	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05

n1: Number of mice with certain viscera metastasis; n2: Number of mice which developed intra-abdominal metastases after inoculation of SW1116 cells.

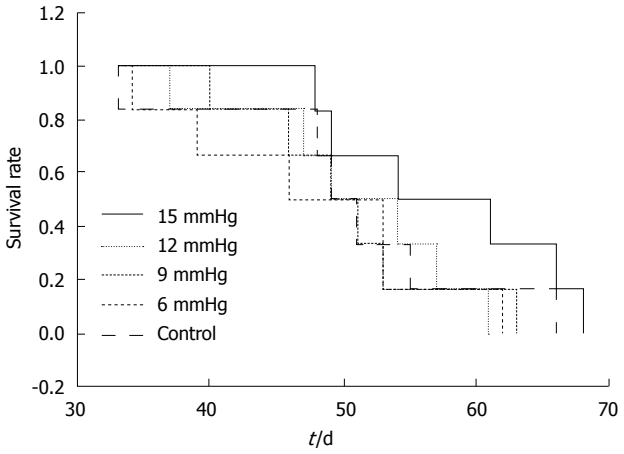

Figure 7 Survival time of the mice injected with SW1116 cells in the different groups ($P = 0.426$).

the expression of P-cadherin mRNA in the mouse peritoneum. However, neither of these two groups investigated how these CO_2-induced changes affected the invasive and metastatic potential of colon cancer cells.

In our study, we found that the expression of these adhesion molecules in the SW1116 cell line increased significantly 0-48 h after the cells were exposed to continuous CO_2-insufflation for 1 h ($P < 0.05$), and returned to the control level by 72 h. We found that these changes are temporary. At certain time points, ICAM-1, E-selectin, and CD44 were found to be even lower than the control levels ($P < 0.05$). In addition, contrary to the results reported by Kim *et al*[16], the expression of E-cadherin significantly increased, rather than decreased after exposure to CO_2-insufflation, although the effect was temporary. The important findings of the present study are that the changes in these adhesion molecules are bi-directional. Collectively increased expression of ICAM-1, E-selectin, and CD44 and decreased expression of E-cadherin signify increasing potential for tumor metastasis. However, the results of the present study also demonstrated the temporary and bi-directional nature of the changes in the expression of these adhesion molecules, indicating that CO_2-insufflation during laparoscopic surgery does not appear to increase the metastatic potential of solid

cancers. Our results document that with increasing CO_2-insufflation pressure, the expression of E-cadherin, CD44 and ICAM-1 decreased, especially at the start of CO_2-insufflation exposure ($P < 0.05$, Figure 3A-C). We believe that higher pressures of CO_2-insufflation could inhibit the expression of adhesion molecules.

We also investigated the effect of CO_2-insufflation on the adhesion and invasion potential of colon cancer cells *in vitro* and the effect on the metastatic capacity of the tumor cells *in vivo*. The cell invasion assays showed that CO_2-insufflation also changed the invasion potential of tumor cells (both the SW1116 and the Lovo cell line) temporarily but with increasing CO_2-insufflation pressures, invasion potential decreased. Similar results were obtained with the adhesion assays.

The *in vivo* animal studies with rising CO_2-insufflation pressure, showed a decrease in the number of tumor nodules when the data between the 15 mmHg and 6 mmHg groups were compared. This observation suggests that high CO_2-insufflation pressures could inhibit the adhesion, invasion and metastatic potential of colon cancer cells. Ziprin *et al*[28] reported that laparoscopic enhancement of tumor cell binding to the peritoneum is inhibited by anti-ICAM-1 monoclonal antibody. High CO_2-insufflation pressures could inhibit the adhesion, invasion or metastatic capacity of tumor cells *in vitro* and *in vivo* by inhibiting expression of the adhesion molecules ICAM-1 and CD44.

In the present study, the adhesion potential of the tumor cells from the CO_2-insufflation groups did not increase when compared to the control group. In addition, the numbers of tumor nodules in the four CO_2-insufflation groups were similar to the control group. Likewise, survival rates were not significantly different indicating that CO_2-insufflation did not increase the adhesion potential of colon cancer cells *in vitro* or the metastatic capacity *in vivo*. However, reaction of the host seems also to be highly important. Various host defence mechanisms in the organism are involved in tumouricidal killing processes. Some studies have reported that the mononuclear phagocytotic system, which largely consists of hepatic tissue macrophages (Kupffer cells), plays an important tumouricidal role. Previously, laparoscopic insufflation with CO_2 had been demonstrated to impair the activity of the mononuclear phagocyte system and was responsible for potential hepatic disadvantages[29]. In this study, only tumor cells treated with CO_2-insufflation *in vitro*, and the *in vivo* metastatic capacity of colon cancer cells treated with different CO_2-insufflation *in vitro* were the focus of this investigation. The reaction of the host after high pressure pneumoperitoneum, and the effect that the pneumoperitoneum may have on intraperitoneal cells was not considered. Further investigations are required.

In conclusion, this study has shown that the expression of adhesion molecules can be affected temporarily and bi-directionally by continuous CO_2-insufflation, which also induced a temporary change in the adhesion and invasion capacity of these cells *in vitro*. CO_2-insufflation at high pressures plays an important role in inhibiting the expression of adhesion molecules. In addition, high insufflation pressures inhibited the adhesion and invasion potential of colon cancer cells *in vitro* and inhibited metastatic potential *in vivo*, which was associated with inhibited expression of adhesion molecules. The results of our study do not provide any evidence that CO_2-insufflation increases the metastatic potential of colon cancer cells.

In conclusion, CO_2-insufflation induced a temporary change in the adhesion and invasion capacity of cancer cells *in vitro*. High pressures of CO_2-insufflation inhibited the adhesion, invasion and metastatic potential *in vitro* and *in vivo*, which was associated with reduced expression of adhesion molecules.

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COMMENTS

Background

Laparoscopic CO_2-insufflation has been reported to correlate with growth and metastasis of colorectal cancer. However, few studies have addressed the influence of CO_2-insufflation pressure on the biological behaviour of colorectal cancer. The aim of this study is to investigate the influence of CO_2-insufflation pressure on adhesion, invasion and metastatic potential of colon cancer cells based on adhesion molecules expression.

Research frontiers

Recently, it has been reported that the CO_2 insufflation can effect the expression of some adhesion molecules, such as E-cadherin, CD44 and ICAM-1, which are related to the tumor metastasis. But few studies concerning the influence of the CO_2-insufflation pressure on the adhesion molecules and how CO_2 insufflation further impacts on the metastatic potential following altered expression of these adhesion molecules.

Innovations and breakthroughs

In this study, the authors found that CO_2-insufflation induced a temporary change in the adhesion and invasion capacity of cancer cells *in vitro*. High pressures of CO_2-insufflation inhibited the adhesion, invasion and metastatic potential *in vitro* and *in vivo*, which was associated with reduced expression of adhesion molecules.

Applications

The results of their study do not provide any evidence that CO_2-insufflation increases the metastatic potential of colon cancer cells. CO_2-insufflation may be used with oncological safety in laparoscopic surgery for colorectal cancer.

Peer review

The manuscript by Jun-Jun Ma and co-authors is well presented, and deals with a significant clinical issue when tumor tissue is resected. Indeed reoccurrences of tumor nodes stand central as a major post-operative complication. Furthermore, the authors applied an original approach to study this problem *in vitro* and *in situ*. This paper contains important information which might have significant translational medical implications.

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Biochemical metabolic changes assessed by ^{31}P magnetic resonance spectroscopy after radiation-induced hepatic injury in rabbits

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Abstract

AIM: To compare the features of biochemical metabolic changes detected by hepatic phosphorus-31 magnetic resonance spectroscopy (^{31}P MRS) with the liver damage score (LDS) and pathologic changes in rabbits and to investigate the diagnostic value of ^{31}P MRS in acute hepatic radiation injury.

METHODS: A total of 30 rabbits received different radiation doses (ranging 5-20 Gy) to establish acute hepatic injury models. Blood biochemical tests, ^{31}P MRS and pathological examinations were carried out 24 h after irradiation. The degree of injury was evaluated according to LDS and pathology. Ten healthy rabbits served as controls. The MR examination was performed on a 1.5 T imager using a ^{1}H/^{31}P surface coil by the 2D chemical shift imaging technique. The relative quantities of phosphomonoesters (PME), phosphodiesters (PDE), inorganic phosphate (Pi) and adenosine triphosphate (ATP) were measured. The data were statistically analyzed.

RESULTS: (1) Relative quantification of phosphorus metabolites: (a) ATP: there were significant differences (P < 0.05) (LDS-groups: control group *vs* mild group *vs* moderate group *vs* severe group, 1.83 ± 0.33 *vs* 1.55 ± 0.24 *vs* 1.27 ± 0.09 *vs* 0.98 ± 0.18; pathological groups: control group *vs* mild group *vs* moderate group *vs* severe group, 1.83 ± 0.33 *vs* 1.58 ± 0.25 *vs* 1.32 ± 0.07 *vs* 1.02 ± 0.18) of ATP relative quantification among control group, mild injured group, moderate injured group, and severe injured group according to both LDS grading and pathological grading, respectively, and it decreased progressively with the increased degree of injury (r = -0.723, P = 0.000). (b) PME and Pi; the relative quantification of PME and Pi decreased significantly in the severe injured group, and the difference between the control group and severe injured group was significant (P < 0.05) (PME: LDS-control group *vs* LDS-severe group, 0.86 ± 0.23 *vs* 0.58 ± 0.22, P = 0.031; pathological control group *vs* pathological severe group, 0.86 ± 0.23 *vs* 0.60 ± 0.21, P = 0.037; Pi: LDS-control group *vs* LDS-severe group, 0.74 ± 0.18 *vs* 0.43 ± 0.14, P = 0.013; pathological control group *vs* pathological severe group, 0.74 ± 0.18 *vs* 0.43 ± 0.14, P = 0.005) according to LDS grading and pathological grading, respectively. (c) PDE; there were no significant differences among groups according to LDS grading, and no significant differences between the control group and experimental groups according to pathological grading. (2) The ratio of relative quantification of phosphorus metabolites: significant differences (P < 0.05) (LDS-moderate group and LDS-severe group *vs* LDS-control group and LDS-mild group, 1.94 ± 0.50 and 1.96 ± 0.72 *vs* 1.43 ± 0.31 and 1.40 ± 0.38) were only found in PDE/ATP between the moderate injured group, the severe injured group and the control group, the mild injured group. No significant difference was found in other ratios of relative quantification of phosphorus metabolites.

CONCLUSION: ^{31}P MRS is a useful method to evaluate early acute hepatic radiation injury. The relative quantification of hepatic ATP levels, which can reflect the pathological severity of acute hepatic radiation injury, is correlated with LDS.

Key words: Liver; Magnetic resonance spectroscopy; Animal models; Pathology; Adenosine triphosphate

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Yu RS, Hao L, Dong F, Mao JS, Sun JZ, Chen Y, Lin M, Wang ZK, Ding WH. Biochemical metabolic changes assessed by ^{31}P magnetic resonance spectroscopy after radiation-induced hepatic injury in rabbits. *World J Gastroenterol* 2009; 15(22): 2723-2730 Available from: URL: http://www.wjgnet.com/1007-9327/15/2723.asp DOI: http://dx.doi.org/10.3748/wjg.15.2723

INTRODUCTION

Acute hepatic radiation injury can lead to necrosis of hepatocytes, fatty degeneration and hepatic fibrosis. The current gold standard test is liver biopsy. This procedure is invasive, uncomfortable for the patient and sometimes leads to serious complications. These factors highlight the need for a noninvasive test to characterize diffuse liver disease. Already, it has been reported that phosphorus-31 magnetic resonance spectroscopy (^{31}P MRS) not only complements liver biopsy but also is a possible replacement and, furthermore, ^{31}P MRS has a particular value in assessing disease progression[1].

^{31}P MRS has been used to study liver metabolism *in vivo* for several years, including clinical liver disease[1-5] and experimental studies[6-8]. It enables the observation of energy metabolism through the signals of phosphomonoesters (PME), phosphodiesters (PDE), inorganic phosphate (Pi) and adenosine triphosphate (ATP). The PME and PDE signals are multi-component, with phosphorylcholine and phosphorylethanolamine the main contributors to PME as well as glycerophosphorylcholine and glycerophosphorylethanolamine which are the main contributors to PDE[1]. The final typical signal of ^{31}P MR spectra *in vivo* is phosphocreatine (PCr). Although it is a dominant signal in muscles, it is not readily observable in spectra of the liver because of its small contribution to hepatic metabolic processes. Its presence indicates some contribution of signals from abdominal wall muscles as a partial volume effect.

In this study, we investigated whether changes of ^{31}P MRS in the liver with early acute radiation injury were related to the liver damage score (LDS) and pathologic changes, we determined the value of ^{31}P MRS in detecting early acute hepatic radiation injury, and we identified the most valuable phosphorylated metabolite for detecting acute hepatic injury. This study set out to provide a rationale for clinical application of ^{31}P MRS in diffuse liver disease.

MATERIALS AND METHODS

Hepatic radiation injury model and experimental groups

This study was approved by the Animal Care Committee of Zhejiang University, School of Medicine. Forty healthy adult New Zealand white rabbits weighing 2.5-3.0 kg were used. These rabbits were randomly assigned into four groups of 10 rabbits. (1) Control group: without any treatment; (2) Group 1: the hepatic region of each rabbit received a single 5 Gy dose of radiation using an 8 MeV electron beam; (3) Group 2: the hepatic region of each rabbit received a single 10 Gy dose of radiation using an 8 MeV electron beam; (4) Group 3: the hepatic region of each rabbit received a single 20 Gy dose of radiation using an 8 MeV electron beam. The irradiation was confined to the whole liver by imaging-guidance. Blood biochemical tests and ^{31}P MRS were carried out 24 h after irradiation. Following each MRS examination, animals were sacrificed, and the liver samples were collected for pathological examination.

MRS examination and spectra evaluation

MRS examination was performed on a Siemens Sonata (Erlangen, Germany) whole-body MR imager operating at 1.5 Tesla equipped with a commercial dual ^{1}H/^{31}P surface coil. Prior to MR examination, animals were fasted overnight. A skin mark in the center of the hepatic region was used in each rabbit at first examination to reduce error. All MR examinations were performed between 8:00 am and 12:00 noon and animals were anesthetized with pentothal sodium (the depth of anesthesia kept well under control) and placed in a prone position with the liver centered on the surface coil.

The basic MR images in all orientations were obtained with true fast imaging with steady precession (true FISP) sequence for the localization of voxels. ^{31}P MR spectra were measured using a standard 2-dimensional chemical shift imaging (CSI) technique[9] in the transverse plane with the following parameters: TR = 440 ms, TE = 2.3 ms, matrix 8 × 8, viewing interpolation 16 × 16, field of view = 200 mm, mean number of times = 120, flip angle = 90 degrees, thickness = 4 cm, voxel volume 2.5 cm × 2.5 cm × 4 cm, acquisition time = 7.36 min. Respiration gating was not used.

Spectra were evaluated using Siemens syngo 2004B software. Briefly, the free induction decay underwent 10 kHz exponential line broadening prior to Fourier transformation, and the resulting spectra were processed with manual phase and baseline correction. Peaks were registered relative to α-ATP resonance (-7.5 ppm), which served as an internal chemical shift reference. Finally, peak integrals were calculated by Gaussian curve fitting with all signals treated as singlets. Signal intensities of PME, Pi, PDE and β-ATP (α-ATP and γ-ATP signals

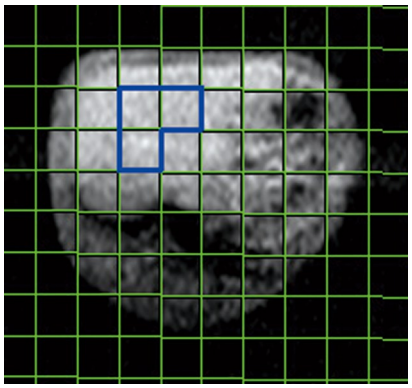

Figure 1 Orientation of CSI: the location of VOI of three voxels being selected in the largest section of rabbit liver on MRI with true FISP sequence.

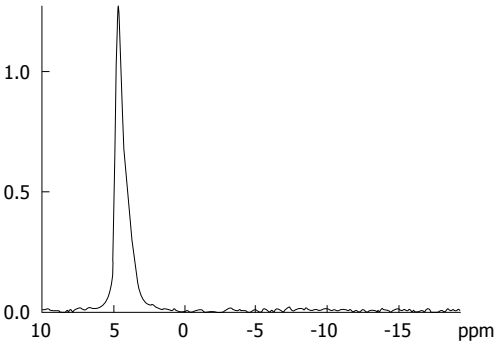

Figure 2 ^{31}P magnetic resonance spectrum from a 500 mL phosphate (NaH_2PO_4) solution phantom with 0.05 mol/L concentrations.

were not used for the evaluation because of overlap with signals of other compounds), which are derived from the integral values of peaks on the spectra, were used for the measurement of relative quantification of metabolites. Volume of interest (VOI) for quantitative evaluation was selected in the center of the liver and the mean integral value of peaks of three voxels on the spectra was used for quantification of metabolites for a rabbit to reduce error (Figure 1).

Phantom experiments were performed before the detection of relative quantification of hepatic metabolites in each rabbit to reduce error induced by the MR imager and environment factors[6,10]. A 500 mL phosphate (NaH_2PO_4) solution phantom with 0.05 mol/L concentration served as a phantom, on which identical MRS examinations were performed regularly throughout the experiment (Figure 2).

For the phantom, the relative quantification of phosphate was 16.6 ± 0.5, and the coefficient of variation was 3.03% (0.5/16.6). The ratio of relative quantification of phosphate between 2 d was conducted as the MRS correction factor (CF) of our MR imager, which was used to correct the relative quantification of hepatic metabolites in each rabbit. Therefore, all the relative quantification of phosphorus metabolites was corrected relative quantification. (corrected relative quantification = relative quantification × CF).

The corrected relative quantification might decrease the error made by the MR imager and the environmental factor in the room, and guarantee the comparability of various relative quantification of phosphorus metabolites.

Table 1 The criteria of liver damage score

Grade	Albumin (g/L)	γ-globulins (g/L)	AST (U/L)	Conjugated bilirubin (μmol/L)	Creatinine (μmol/L)
0	> 36.5	< 19.9	< 50	< 6	< 119
1	32.9-36.4	20-26	51-180	7-32	120-150
2	28.5-32.8	26.1-34.9	181-384	33-75	151-230
3	24.5-28.4	> 35	> 385	> 76	> 231
4	21.8-24.4				
5	< 21.7				

Evaluation of the degree of injury

This study adopted two methods to evaluate the degree of injury: LDS grading and pathological grading.

LDS grading: Sera were isolated from collected blood samples, and serum aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, r-glutamyl transpeptidase, albumin, globulin and albumin/globulin levels were measured by Olympus 2700 analyzer. Then the LDS was calculated for each rabbit according to Krastev's standard (Table 1)[11]. The degree of injury of the liver was divided into mild (LDS ⩽ 3 U), moderate (LDS 3-6 U) and severe (LDS > 6 U).

Pathological grading: The paraffin-section method and hematoxylin and eosin stain was applied to all liver samples, which were read by a single independent liver pathologist (Dr. Lin M) and assessed for swelling, degeneration, necrosis of hepatocytes, and hepatic hemorrhage. The pathologist was blinded to dose of radiation received and the results of ^{31}P MRS. (1) Normal group (control group): normal hepatocytes, integrity of structure of hepatic lobules and regular arrangement of hepatic cord (Figure 3A). (2) Mild group: mild cellular swelling, fatty degeneration and (or) hydropic degeneration, without cell necrosis or hepatic hemorrhage (Figure 3B). (3) Moderate group: moderate cellular swelling and fatty degeneration accompanied by punctal necrosis and stray bleeding points (Figure 3C). (4) Severe group: diffuse cellular swelling and fatty degeneration, with constriction or emphraxis of hepatic sinuses, with or without local cell necrosis and (or) hepatic hemorrhage (Figure 3D).

Statistical analysis

The data were expressed as mean ± SD. Analysis of variance with SNK tests (the Student-Newman-Keuls post-hoc tests) of one-way ANOVA with SPSS 11.0 was used to examine differences between groups. Using Pearson's correlation test, the correlation between relative quantification of ATP and LDS was examined. $P < 0.05$ were considered to indicate statistical significance.

RESULTS

Analysis of hepatic phosphorylated metabolite levels

For the control group, all rabbits had normal hepatic

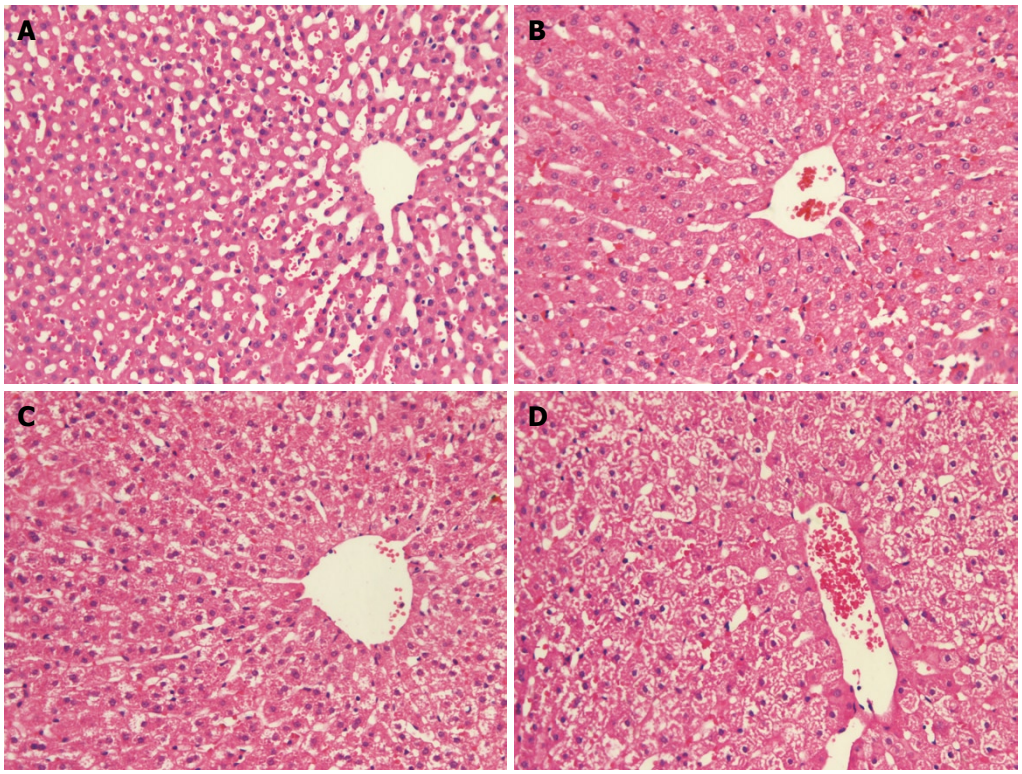

Figure 3 Hepatic ^{31}P MRS of the liver from a rabbit of the control group (A), mild injury group (B), moderate injury group (C) and severe injury group (D). A significant decrease in the ATP signal is seen in the ^{31}P MR spectra (C, D).

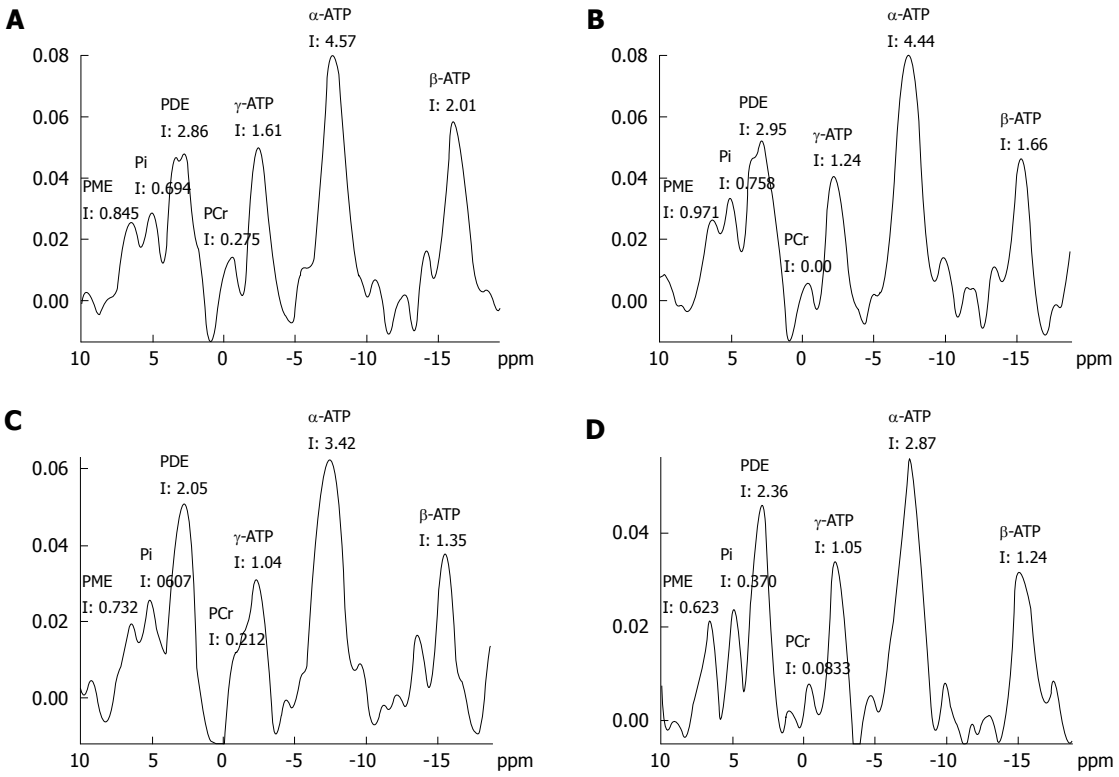

Figure 4 Representative histological section of the liver from a rabbit of the control group (A), mild injury group (B), moderate injury group (C) and severe injury group (D). I: Integral.

micro-structure and a LDS of zero, while rabbits in the experimental groups varied between each other in pathology and LDS, depending on the degree of injury. The spectroscopic data of rabbits in the control group and rabbits with acute hepatic radiation injury, grouped by LDS are summarized in Table 2. The spectroscopic data of rabbits in the control group and rabbits with acute hepatic radiation injury, grouped by pathology are summarized in Table 3. The relative quantification of phosphorus metabolites detected by MRS included PME, PDE, Pi and ATP (Figure 4).

The preceding two tables (Tables 2 and 3) showed

Table 2 Relative quantification of metabolites in rabbit liver using the LDS evaluation method (mean ± SD)

Metabolite	Control group (n = 10)	LDS-mild group (n = 13)	LDS-moderate group (n = 9)	LDS-severe group (n = 8)	F	P
PME	0.86 ± 0.23[j]	0.79 ± 0.33	0.80 ± 0.22	0.58 ± 0.22[a]	1.852	0.155
PDE	2.27 ± 0.62	2.14 ± 0.51	2.48 ± 0.63	1.99 ± 0.88	3.023	0.042
Pi	0.74 ± 0.18[j]	0.63 ± 0.28	0.62 ± 0.29	0.43 ± 0.14[a]	3.475	0.026
ATP	1.83 ± 0.33[e,i,l]	1.55 ± 0.24[b,h,l]	1.27 ± 0.09[c,e,j]	0.98 ± 0.18[c,f,g]	22.647	0.000
PME/PDE	0.36 ± 0.12	0.39 ± 0.18	0.35 ± 0.14	0.44 ± 0.54	0.462	0.710
PME/ATP	0.50 ± 0.11	0.50 ± 0.18	0.62 ± 0.15	0.61 ± 0.25	0.990	0.409
PDE/ATP	1.43 ± 0.31[g,j]	1.40 ± 0.38[g,j]	1.94 ± 0.50[a,d]	1.96 ± 0.72[a,d]	5.080	0.005
Pi /ATP	0.40 ± 0.09	0.41 ± 0.19	0.49 ± 0.24	0.46 ± 0.16	1.582	0.211
PME/Pi	1.47 ± 0.65	1.49 ± 0.92	1.76 ± 1.12	1.39 ± 0.58	0.081	0.970
PDE/Pi	3.46 ± 1.15	4.26 ± 2.58	4.91 ± 2.65	5.05 ± 3.18	0.902	0.450

[a]P < 0.05, [b]P < 0.01, [c]P < 0.005 *vs* control group; [d]P < 0.05, [e]P < 0.01, [f]P < 0.005 *vs* mild group; [g]P < 0.05, [h]P < 0.01, [i]P < 0.005 *vs* moderate group; [j]P < 0.05, [k]P < 0.01, [l]P < 0.005 *vs* severe group.

Table 3 Relative quantification of metabolites in rabbit liver with the pathological evaluation method

Metabolite	Control group (n = 10)	Pathological mild group (n = 12)	Pathological moderate group (n = 11)	Pathological severe group (n = 7)	F	P
PME	0.86 ± 0.23[j]	0.82 ± 0.35	0.79 ± 0.24	0.60 ± 0.21[a]	1.861	0.154
PDE	2.27 ± 0.62	2.08 ± 0.47[g]	2.67 ± 0.38[d,k]	1.92 ± 0.83[h]	1.118	0.355
Pi	0.74 ± 0.18[k]	0.61 ± 0.24	0.7 ± 0.33[j]	0.43 ± 0.14[b,g]	2.334	0.090
ATP	1.83 ± 0.33[d,i,l]	1.58 ± 0.25[a,g,l]	1.32 ± 0.07[c,d,k]	1.02 ± 0.18[c,f,h]	22.878	0.000
PME/PDE	0.36 ± 0.12	0.40 ± 0.18	0.31 ± 0.13	0.45 ± 0.48	0.189	0.903
PME/ATP	0.50 ± 0.11[h]	0.51 ± 0.19[i]	0.60 ± 0.18[b,f]	0.59 ± 0.22	1.412	0.255
PDE/ATP	1.43 ± 0.31[h]	1.34 ± 0.33[i,j]	2.04 ± 0.36[b,f]	1.83 ± 0.72[d]	4.082	0.014
Pi/ATP	0.40 ± 0.09	0.39 ± 0.15	0.54 ± 0.26	0.43 ± 0.16	0.601	0.619
PME/Pi	1.47 ± 0.65	1.57 ± 0.97	1.44 ± 1.03	1.61 ± 0.75	0.316	0.814
PDE/Pi	3.46 ± 1.15	4.12 ± 2.36	4.87 ± 2.63	5.09 ± 3.24	0.810	0.497

[a]P < 0.05, [b]P < 0.01, [c]P < 0.005 *vs* control group; [d]P < 0.05, [e]P < 0.01, [f]P < 0.005 *vs* mild group; [g]P < 0.05, [h]P < 0.01, [i]P < 0.005 *vs* moderate group; [j]P < 0.05, [k]P < 0.01, [l]P < 0.005 *vs* severe group.

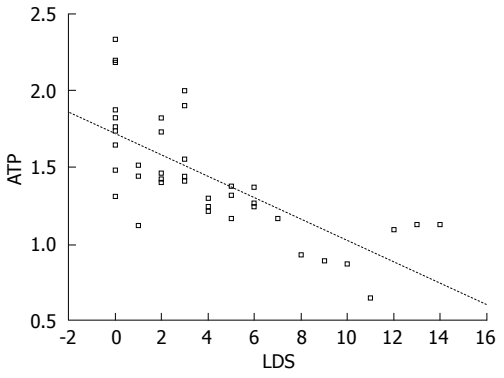

Figure 5 The graph shows the correlation between LDS and ATP, that is, the ATP relative quantification decreases progressively with the increase of LDS.

the relation between biochemical index and relative quantification of phosphorus metabolites, as well as the relation between pathology and relative quantification of phosphorus metabolites. However, the results evaluated with LDS and pathology were of perfect consistency. The analysis is detailed below.

Characteristics of relative quantification of hepatic phosphorus metabolites

ATP: There were significant differences in ATP relative quantification among the control group, mild group, moderate group, and severe group according to both LDS grading and pathological grading (LDS groups: mild group, moderate group, severe group *vs* control group had P = 0.007, 0.000, 0.000, respectively; moderate group, severe group *vs* mild group had P = 0.008, 0.000, respectively; severe group *vs* moderate group had P = 0.013. Pathological groups: mild group, moderate group, severe group *vs* control group had P = 0.017, 0.000, 0.000, respectively; moderate group, severe group *vs* mild group had P = 0.000, 0.000, respectively; severe group *vs* moderate group had P = 0.008); it decreased progressively with the increased degree of injury, which is visually displayed by the correlation graph of ATP and LDS (Figure 5). These data illustrated that the hepatic ATP level may be the most sensitive criterion for reflecting hepatic injury in rabbits.

PME and Pi: The relative quantification of PME and Pi decreased significantly in the severe injured group, and the difference was significant compared with the control group (PME: LDS-severe group *vs* LDS-control group had P = 0.031, pathological-severe group *vs* pathological-control group had P = 0.037. Pi: LDS-severe group *vs* LDS-control group had P = 0.013, pathological-severe group *vs* pathological-control group had P = 0.005). Also, from the ^{31}P MR spectra (Figure 4C and D), a significant decrease in the signal of phosphorylated metabolites could be seen. This indicated that if there was a significant

difference in PME and Pi between normal data and test data, the tested liver was likely to be severely injured.

PDE: The relative quantification of various hepatic PDE levels changed irregularly, which indicated that the relative quantification levels of PDE may not be applied solely to assess acute hepatic radiation injury.

The characteristics of the ratio of relative quantification of other phosphorus metabolites

There were significant changes in the PDE/ATP ratio between control group and moderate group; mild group and moderate, severe group; moderate group and control group, mild group (LDS-groups: moderate group, severe group *vs* control group had $P = 0.026$, 0.025, respectively; moderate group, severe group *vs* control group had $P = 0.014$, 0.014, respectively; pathological-groups: moderate group, severe group *vs* control group had $P = 0.076$, 0.064, respectively; moderate group, severe group *vs* control group had $P = 0.002$, 0.020, respectively). There were no significant changes in the PDE/Pi ratio among all groups. Compared with the control group, no significant changes of PME/PDE, Pi/ATP and PME/Pi ratio were found in other groups.

The above results illustrated that there were few characteristic differences in the ratios of relative quantification of various hepatic phosphorus metabolites in hepatic radiation injury. Therefore, the ratios of relative quantification may not be used to evaluate acute mild hepatic radiation injury.

DISCUSSION

Acute liver diseases can result from various causes which operate through different pathophysiological pathways and which elicit distinct patterns of hepatic injury. Diagnosis of acute liver diseases including hepatic radiation injury is mainly based on invasive methods such as liver biopsy, laparoscopy, various radiological examinations and other clinical tests. On the other hand, signals from ^{31}P MRS reflect *in vivo* intracellular and membrane metabolism non-invasively and they are objective parameters[2,12].

Evaluation method for ^{31}P MRS and its influencing factors

The evaluation of ^{31}P MRS in the liver includes absolute or relative quantification of metabolite levels. Nowadays, absolute quantification of metabolites in mmol/L is hampered by the use of too short TR values and other technical complications[13,14], but most studies with ^{31}P MRS deal with the relative signal intensity for quantification. Also, here we measured the relative quantification of metabolites, and the relative signal ratios of the metabolites. The relative quantification evaluation had to satisfy 3 conditions for decreasing detection errors: (1) phantom experiments before relative quantification of hepatic metabolites in each rabbit to reduce errors induced by the MR imager and environment factors, *etc*; (2) a VOI selected in the largest section of liver and a small PCr signal characterizing the presence of abdominal muscles; (3) moderate anesthesia of rabbits and keeping the rabbits in the same position in the bed of the MR imager.

The changes in hepatic ATP levels

Many published studies of ^{31}P MR spectra have shown that acute and chronic diffuse liver diseases are associated with a reduction in hepatic ATP levels[3,6,7], and some studies found that changes in hepatic ATP levels correlate with changes in liver histology[3,6]. Our findings, that the relative quantification of hepatic ATP levels displayed progressive reductions with increased hepatic injury, correspond to the results of a previous study[6], but we are the first to report that the changes in hepatic ATP levels correlate with the severity of acute hepatic radiation injury. Some reports have shown that, during the early phase of chronic diffuse liver diseases, only minor changes in hepatic ATP could be detected[15,16]. Our study showed that the relative quantification of hepatic ATP levels obviously decreased, because there were significant differences between the control group and both the pathological-mild group and LDS-mild group. We also found that the changes in levels of other hepatic metabolites were less sensitive than the changes in ATP levels in mild hepatic injury, and the relative quantification of hepatic ATP levels could be well correlated with LDS. Thus, we suggest that the hepatic ATP level may be the most reliable criterion for evaluating acute hepatic injury in rabbits. The reason is that the β-ATP peak is unique, and quite different from other phosphorylated metabolite peaks, which overlap with signals of other metabolites in the ^{31}P MRS map.

The mechanisms responsible for the decline in hepatic ATP levels include: gradual loss of viable hepatocytes, which is likely to be an important contributing factor-as the total amount of these cells per unit volume of liver decreases, MRS detectable signal from that volume will also decrease[6]; anoxemia of local liver tissue induced by injury of capillary vessels after hepatic radiation[17]; increased energy expenditure as liver disease progresses[18]. In addition, disturbed hepatic bioenergetics has also been ascribed to the capillarization of hepatic sinusoids during the development of cirrhosis[19].

The changes in levels and ratios of relative quantification of other phosphorus metabolites

PME, PDE and the correlation ratio: information about phospholipid membrane metabolism may also be obtained from the PME and PDE resonances in the ^{31}P MR spectrum. Both resonances are multicomponent peaks containing contributions from several metabolites[20]. The significance of the changes in PME and PDE levels is not clear. Some previous studies have reported that an increase in PME levels is accompanied by a decrease in PDE levels or increased ratios of hepatic PME/ATP and PME/PDE in acute and chronic diffuse liver diseases[1,2,21,22]. However, in other investigators, and our study, PME levels did not increase nor did PDE levels decrease in the same acute and chronic diffuse

liver diseases[6,8]. The main reason for these conflicting findings may be the broad, overlapping characteristics of these peaks along with the multiple signals contributing to these resonances hindering accurate quantification of the PME and PDE peaks[8]. Another explanation is that hepatic phospholipid membrane activity may differ in animal models of liver diseases versus liver diseases in humans[6]. Therefore, either the changes in PME and PDE levels or the ratio PME/PDE cannot accurately reflect the liver diseases.

Pi and the correlation ratio: Pi is another marker of tissue bioenergetics. Increases in Pi have been observed by ^{31}P MRS during high energy activities such as liver regeneration following partial hepatectomy[8,23]. The increase in hepatic Pi may result from the hydrolysis of high energy phosphate bonds which in turn liberates Pi species, increases hepatic uptake and accumulation of Pi due to enhanced metabolic activity and reduces recycling back to purine/pyrimidine moieties[8]. Some previous studies have reported a decrease or no difference in Pi levels in chronic diffuse liver diseases in humans and animals[1,6]. The reason for no changes in hepatic Pi is not clear, but the decrease in hepatic Pi may result from reduced hepatocyte mass[6]. In this study, significant decreases in Pi were only detected among the most seriously injured livers of the severe group. The lower levels of hepatic Pi likely result from reduced hepatocyte mass, as Corbin *et al*[6] reported. In addition, compared with the ATP peak, the Pi peak, which is located between the PME and PDE peaks, is more prone to being impacted by the overlapping characteristics of PME and PDE peaks, hindering accurate quantification of the Pi peak. However, some authors regarded the ratio ATP/Pi as the criterion of hepatic regenerative activity following partial hepatectomy, because the ATP levels decreased, the Pi levels increased, and the changes of the ratio were more sensitive than ATP or Pi alone[8]. However, the levels of ATP or Pi were decreased to different degrees after acute hepatic radiation injury in our study, so the ratio of ATP/Pi could not be used to evaluate acute hepatic radiation injury.

This study illustrated that ^{31}P MRS of clinical 1.5T MRS could detect various changes of phosphorylated metabolite levels in early acute hepatic injury. In addition, the study also showed that though there were many hepatic phosphorylated metabolites and correlated ratios, the measurement of levels of hepatic ATP may be the most reliable criterion for reflecting both pathological hepatic injury and LDS in rabbits.

COMMENTS

Background

Acute hepatic radiation injury can lead to necrosis of hepatocytes, fatty degeneration and hepatic fibrosis. The current gold standard test is liver biopsy. This procedure is invasive, uncomfortable for the patient and sometimes has serious complications. These factors highlight the need for a noninvasive test to characterize diffuse liver disease. Already, it has been reported that phosphorus-31 magnetic resonance spectroscopy (^{31}P MRS) not only complements liver biopsy but also is a possible replacement, and furthermore, ^{31}P MRS has particular value in assessing disease progression.

Research frontiers

^{31}P MRS has been used to study liver metabolism *in vivo* for several years, including clinical liver disease studies and experimental studies. The research focus is how to observe the energy metabolism or pathological changes through the signals of phosphorus metabolites.

Innovations and breakthroughs

In this study the authors carefully used two different methods [liver damage score (LDS), and pathology] to evaluate the degree of injury, and then they studied the correlation between MRS and the degree of injury. Furthermore, they report that the changes in hepatic adenosine triphosphate (ATP) levels correlate with the severity of acute hepatic radiation injury measured by LDS.

Applications

This study may be particularly useful for allowing clinical detection of early acute hepatic injury with ^{31}P MRS in the future.

Terminology

MRS: Spectroscopic method for measuring the magnetic moment of elementary particles such as atomic nuclei, protons or electrons. It is employed in clinical applications such as NMR Tomography (magnetic resonance imaging).

Peer review

^{31}P MRS is a very interesting method especially to replace the gold standard biopsy, particularly in assessing disease progression.

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Anti-*Helicobacter pylori* therapy followed by celecoxib on progression of gastric precancerous lesions

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Abstract

AIM: To evaluate whether celecoxib, a selective cyclooxygenase 2 (COX-2) inhibitor, could reduce the severity of gastric precancerous lesions following *Helicobacter pylori* (*H pylori*) eradication.

METHODS: *H pylori*-eradicated patients with gastric precancerous lesions randomly received either celecoxib (n = 30) or placebo (n = 30) for up to 3 mo. COX-2 expression and activity was determined by immunostaining and prostaglandin E_2 (PGE_2) assay, cell proliferation by Ki-67 immunostaining, apoptosis by TUNEL staining and angiogenesis by microvascular density (MVD) assay using CD31 staining.

RESULTS: COX-2 protein expression was significantly increased in gastric precancerous lesions (atrophy, intestinal metaplasia and dysplasia, respectively) compared with chronic gastritis, and was concomitant with an increase in cell proliferation and angiogenesis. A significant improvement in precancerous lesions was observed in patients who received celecoxib compared with those who received placebo (P < 0.001). Of these three changes, 84.6% of sites with dysplasia regressed in patients treated with celecoxib (P = 0.002) compared with 60% in the placebo group, suggesting that celecoxib was effective on the regression of dysplasia. COX-2 protein expression (P < 0.001) and COX-2 activity (P < 0.001) in the gastric tissues were consistently lower in celecoxib-treated patients compared with the placebo-treated subjects. Moreover, it was also shown that celecoxib suppressed cell proliferation (P < 0.01), induced cell apoptosis (P < 0.01) and inhibited angiogenesis with decreased MVD (P < 0.001). However, all of these effects were not seen in placebo-treated subjects. Furthermore, COX-2 inhibition resulted in the up-regulation of PPARγ expression, a protective molecule with anti-neoplastic effects.

CONCLUSION: *H pylori* eradication therapy followed by celecoxib treatment improves gastric precancerous lesions by inhibiting COX-2 activity, inducing apoptosis, and suppressing cell proliferation and angiogenesis.

Key words: Apoptosis; Cyclooxygenase 2; Gastric precancerous lesions; *Helicobacter pylori*; Microvessel density; Proliferation

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INTRODUCTION

Gastric cancer is the second leading cause of cancer deaths worldwide[1] and its 5-year survival rate is only 10%-15% in individuals with advanced disease[2]. *Helicobacter pylori* (*H pylori*) has been classified as a type I carcinogen by the WHO and is recognized as an important pathogen in gastric tumorigenesis[3]. *H pylori* infection initiates the inflammatory and atrophic changes in gastric mucosa accompanied by enhanced expression of some protumorigenic factors such as cyclooxygenase 2 (COX-2) and anti-apoptosis proteins, resulting in uncontrolled proliferation of mutated atrophic cells, suppression of apoptosis, excessive angiogenesis and finally the formation of adenocarcinoma[4].

In cancer prevention, the targeting of precancerous lesions has been recognized as the most promising method. However, progress has been achieved only in the chemoprevention of colorectal neoplasia[5]. There is no effective therapy for reversing gastric premalignant lesions. Although *H pylori* infection is a critical initiator and mediator in gastric premalignant changes and gastric carcinogenesis, eradication of *H pylori* alone failed to improve these precancerous lesions[6-8]. The failure of *H pylori* eradication may be explained by the fact that the expression of COX-2, an important mediator in *H pylori*-induced premalignant changes, remained high or only modestly reduced after *H pylori* eradication[6-8]. It has been widely accepted that COX-2 plays an important role in gastric carcinogenesis[9]. COX-2 over-expression has been found in *H pylori*-induced inflammation, precancerous lesions and gastric tumors[9]. Inhibition of COX-2 by non-steroidal anti-inflammatory drugs (NSAIDs) has been proven to be effective in preventing gastric carcinogenesis as evidenced in animal models and epidemiological studies[2,9,10]. Since eradication of *H pylori* alone is not sufficient to reverse gastric carcinogenesis due to failure of the inhibition of *H pylori*-induced protumorigenic factors such as COX-2, it would be reasonable to combine additional treatments such as COX-2 inhibition following *H pylori* eradication.

In the present study, we examined whether treatment with the selective COX-2 inhibitor celecoxib could reduce the severity of gastric precancerous lesions after *H pylori* eradication. The mechanisms of its action were also investigated.

MATERIALS AND METHODS

Patients and study design

We enrolled 233 patients who had upper-gastrointestinal symptoms such as anorexia, early satiety, stomach pain, abdominal distention and epigastric discomfort between January 2005 and July 2006 from the First Affiliated Hospital of Hebei Medical University, Shijiazhuang, China. Eligible subjects were between 30 and 70 years of age, and had no history of drug allergies. Subjects were ineligible if they were under 30 or older than 70 years old; pregnant or lactating; had peptic ulcer; gastric cancer or other cancers; upper gastrointestinal bleeding; liver cirrhosis; serious cardiovascular diseases, renal or lung diseases; hypersensitive to COX-2 inhibitors, NSAIDs, salicylates, or sulfonamides; used NSAIDs; and those unwilling to undergo repeat endoscopy after treatment.

H pylori infected patients who were defined both by the presence of the bacterium on histology as well as a positive C^{14} urea breath test were treated with a 1-wk course of eradication therapy (omeprazole, 20 mg; amoxicillin, 1000 mg; furaltadone, 100 mg; twice daily). Five weeks post-treatment, patients were recalled to hospital for repeat endoscopy and C^{14} urea breath test. *H pylori* eradication was confirmed by a negative C^{14} urea breath test and a negative *H pylori* histology examination from biopsies. Only patients with confirmed *H pylori* eradication were recruited into this study. During each endoscopy, a total of eight gastric biopsies were taken from the antrum (two from the greater curvature and two from the lesser curvature) and the corpus of the stomach (two from the greater curvature and two from the lesser curvature) for molecular experiments and histological examination. One of the two biopsy specimens from each site was immediately stored at -80℃ for RNA/protein extraction. The other specimens were fixed in 10% neutral buffered formalin and embedded in paraffin for histological examination and immunostaining. The severity of gastric histology including gastritis and precancerous lesions [gastric atrophy, intestinal metaplasia (IM) and low-grade dysplasia] was scored based on the following standards: absent (0), mild (1), moderate (2) and marked (3)[11]. Patients with high-grade dysplasia were excluded from the study. All gastric biopsies were interpreted by two pathologists who were unaware of the treatment assignments.

One hundred and thirty six eligible subjects who were histologically confirmed to have gastric precancerous lesions and negative tests for *H pylori* were randomly assigned to receive either celecoxib 200 mg twice daily or an identical-looking placebo at a 1:1 ratio for 3 mo. During the treatment period, 10 subjects were withdrawn due to adverse events and 66 were lost to follow-up. Some patients lost to follow-up migrated to other cities, some were non-compliant and failed to follow through with our treatment protocol, and some refused to undergo a second endoscopy. At the end of the 3-mo treatment period, 30 patients in the celecoxib group (16 males with an average age of 50.33 ± 10.39 years; 14 females with an average age of 57.36 ± 9.25 years) and 30 patients in the placebo group (14 males with an average age of 48 ± 10.43 years; 16 females with an average age of 50 ± 9.98 years) returned for endoscopic examination. The same protocols for obtaining gastric biopsy and histological examination were used as previously mentioned in the baseline endoscopy. The study protocol was approved by the Clinical Research Ethics Committee and all participants gave written informed consent.

Immunohistochemistry

The paraffin-embedded gastric sections were

Table 1 Primer sequences used for amplification of mRNA by semi-quantitative PCR

mRNA	Forward primer (5′-3′)	Reverse primer (5′-3′)	Size (bp)
PCNA	CTTTTCTGTCACCAAATTTGTACC	AACTGCATTTAGAGTCAAGACCC	206
Fas	CTGCCAAGAAGGGAAGGAGT	GGTGCAAGGGTCACAGTGTT	189
PPARγ	AGCCTCATGAAGAGCCTTCCA	ACCCTTGCATCCTTCACAAGC	89
β-actin	GTCTTCCCCTCCATCGTG	GGGTGAGGATGCCTCTCTT	251

deparaffinized and rehydrated in graded ethanol. The activity of endogenous peroxidase was blocked by methanol containing 3% H_2O_2 for 10 min and washed with PBS. After blocking with 10% nonimmunized goat serum at 37℃ for 20 min, sections were incubated with the primary antibody for COX-2 (1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA), Ki-67 (1:200, Chemicon International Inc., Temecula, CA, USA), peroxisome proliferator-activated receptor γ (PPARγ) (1:100, Santa Cruz) overnight at 4℃. Peroxidase activity was visualized by applying diaminobenzidine to the sections, which were then counter-stained with hematoxylin. Analysis of the immunostained sections was independently performed by two pathologists in a blinded fashion.

Microvessel density (MVD) was performed on paraffin-embedded gastric tissue sections stained with CD31 (1:150, DAKO, Glostrup, Denmark) as an indicator. For the determination of MVD in each case, five of the most highly vascularized areas within a section were selected and counted under a light microscope. The average numbers of microvessels in the selected fields were recorded as the MVD for each case.

cDNA synthesis and RT-PCR

Gastric tissue specimens were homogenized with a homogenizer. Total RNA was extracted using TRIzol reagent (Invitrogen, USA). Five micrograms of total RNA from each sample was reverse transcribed into cDNA using the AMV Reverse Transcription system (Promega, San Luis Obispo, CA, USA). Semi-quantitative PCR was performed. The primer sequences of proliferation cell nuclear antigen (PCNA), factor associated suicide (FAS), PPARγ and β-actin are listed in Table 1. A DNA free template control (containing water) was included and each sample was added in duplicate. PCR products were separated by 15% agarose gel electrophoresis and quantified by the Gel Imaging System after ethidium bromide (10 mg/L) staining. The mRNA expression level of the target gene was defined by the densitometry ratio of target gene to β-actin.

PGE_2 assay

PGE_2 levels were measured in snap frozen tissue specimens using a radioimmunoassay-based assay. Briefly, about 20 mg of snap frozen tissues were homogenized in 10 volumes of sodium chloride by a ground glass homogenizer on ice. The mixture was incubated at 37℃ for 15 min and then centrifuged for 20 min at 3000 r/min. The supernatant was then applied to the pre-primed immunoassay reaction mixture and reacted with the antibody overnight. PGE_2 was precipitated with 0.7 mL volumes of 25% polyethylene glycol. The quantity of PGE_2 in the supernatants was determined using RIA.

Determination of cell proliferation

Proliferation was assayed by immunoperoxidase staining for Ki-67 as described previously[12]. The immunochemistry method for staining the sections with Ki-67 antibody has been specified above. The proliferation index (PI) was defined as a percentage of the ratio of Ki-67-positive nuclei to the total nuclei counted.

Terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL)

Apoptosis was determined *in situ* from paraffin-embedded tissue sections by the TUNEL assay using the *in situ* Cell Death Detection kit (Roche Applied Science, Indianapolis, IN, USA). Briefly, paraffin-embedded slides were deparaffinized, hydrated and incubated with proteinase K (20 mg/mL in 10 mmol/L Tris-HCl) for 20 min. After labeling with the TUNEL reaction mixture, slides were developed by converter-POD and DAB substrate. PBS replaced the primary antibodies as a negative control. The results of staining were analyzed and evaluated by three individuals independently. At least 1000 cells were counted in five random fields. Apoptosis index (AI) was represented as the percentage of positive cells with TUNEL staining to the total cells.

Statistical analysis

Data were analyzed by SPSS 12.0 software and are shown in a default form of mean ± SD. The association between COX-2 expression and the progression of precancerous lesions was analyzed by χ^2 test. The correlation between COX-2 expression and MVD/PI was analyzed using Pearson's Correlation Coefficient. The difference between the groups was compared by *t* test. The paired *t* test was used to determine the difference between pre-treatment and after-treatment within each treatment group. $P < 0.05$ was considered statistically significant.

RESULTS

Association between COX-2 expression and progression of precancerous lesions

The percentage of COX-2 positive cells in gastric tissues was increased with the progression of chronic gastritis

(33.3%), atrophy (51.6%), IM (53.3%), and dysplasia (79.3%) as determined by immunostaining. COX-2 expression in dysplasia (79.3%) was significantly higher than in any other type of lesion ($P < 0.05$). COX-2 expression was significantly elevated in atrophy and IM compared with chronic gastritis ($P < 0.05$). However, there was no significant difference between gastric atrophy and IM ($P > 0.05$).

COX-2 expression correlated with cell proliferation

Cell proliferation was determined by Ki-67 staining. A significant positive correlation between COX-2 expression and PI ($\chi^2 = 10.5$, $P = 0.001$) was observed by Pearson's Correlation Coefficient analysis, indicating COX-2 expression correlated with cell proliferation.

COX-2 expression correlated with angiogenesis

We also evaluated the correlation between COX-2 expression and angiogenesis as determined by CD31 immunostaining. The mean MVD was significantly higher in COX-2-positive tissues ($n = 47$, 23.85 ± 7.44) than in COX-2-negative tissues ($n = 27$, 18.47 ± 6.02) ($P < 0.001$), indicating a positive correlation between COX-2 expression and MVD. Thus COX-2 also played an important role in angiogenesis.

Effect of celecoxib on the improvement in histology of gastric precancerous lesions

After three months treatment, a significant improvement in precancerous lesions was observed in 66.7% (20/30) of patients ($P < 0.001$) who were treated with celecoxib. However, only 16.1% of cases who received placebo showed improved histology ($P > 0.05$). Of these three changes, 84.6% of sites with dysplasia regressed in patients treated with celecoxib ($P = 0.002$) compared with 60% in the placebo group, suggesting that celecoxib was effective on the regression of dysplasia (Table 2). However, differences in pathological improvement of atrophy and intestinal metaplasia were not observed between the celecoxib and placebo groups (Table 2). With regard to the mixed pathological sites with both atrophy and intestinal metaplasia, we did not have sufficient sample size to make an accurate conclusion.

Effect of celecoxib on COX-2 protein expression and COX-2 activity

COX-2 protein expression as determined by the percentage of COX-2 positive cells in the gastric tissues was significantly lower after treatment with celecoxib (pre-treatment: 40.93% ± 11.96% *vs* after-treatment: 27.88% ± 4.94%, $P < 0.001$) (Figure 1A-C). COX-2 protein expression was reduced in 73%, remained the same in 13% and increased in 13% of patients treated with celecoxib. However, COX-2 expression was reduced in only 48% of patients in the placebo group (Table 3). PGE_2 level, an indicator of COX-2 activity, was concomitantly reduced in patients treated with celecoxib (pre-treatment: 358.9 ± 59.3 *vs* after-treatment: 143.6 ± 24.2, $P < 0.001$). However, these differences in COX-2 expression and COX-2 activity were not seen in patients treated with placebo (Figure 1D).

Table 2 Effect of celecoxib on the histological improvement of gastric precancerous lesions

Precancerous lesions	Regression site *n* (%)		*P*-value
	Celecoxib group	Placebo group	
Atrophy	11/13 (84.6)	7/13 (53.8)	0.202
Metaplasia	9/14 (64.3)	9/18 (50)	0.419
Atrophy + metaplasia[1]	3/4 (75)	3/6 (50)	0.895
Dysplasia	35/39 (89.7)	24/40 (60)	0.002

[1]Indicates that both atrophy and metaplasia were regressed in one site.

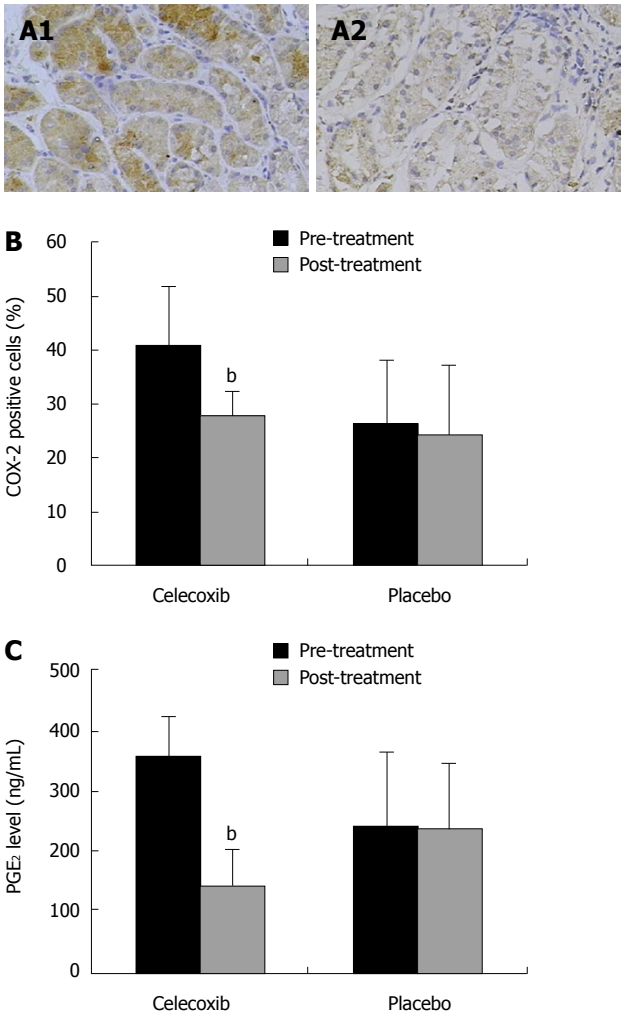

Figure 1 Effects of celecoxib on COX-2 expression and PGE_2 levels. A: Representative image of COX-2 protein expression as determined by immunostaining in paraffin-embedded gastric tissue sections: (A1) pre-treatment, and (A2) post-treatment with celecoxib; B: Percentage of COX-2 positive cells in gastric mucosa; C: PGE_2 levels. Data are mean ± SD. $^bP < 0.001$.

Effect of celecoxib on cell proliferation

Cell proliferation was significantly reduced in the celecoxib group after 3 mo of treatment (pre-treatment: 27.46% ± 6.77% *vs* after-treatment: 20.18% ± 4.05%, $P < 0.01$) (Figure 2A and B). However, this difference was not observed in patients treated with placebo (Figure 2B). Seventy percent of patients in the celecoxib

Table 3 Effect of celecoxib on COX-2 expression and other parameters

	Celecoxib group *n* (%)			Placebo group *n* (%)		
	Reduction	Same	Worse	Reduction	Same	Worse
COX-2 expression	22 (73)	4 (13)	4 (13)	15 (48)	3 (10)	13 (43)
Cell proliferation	23 (77)	2 (7)	5 (17)	11 (37)	3 (10)	16 (53)
Cell apoptosis	8 (53)	4 (27)	3 (20)	4 (27)	5 (17)	6 (40)
Angiogenesis	22 (73)	4 (13)	4 (13)	12 (39)	10 (32)	9 (29)
PPARγ expression	15 (50)	5 (17)	10 (33)	9 (29)	5 (16)	17 (55)

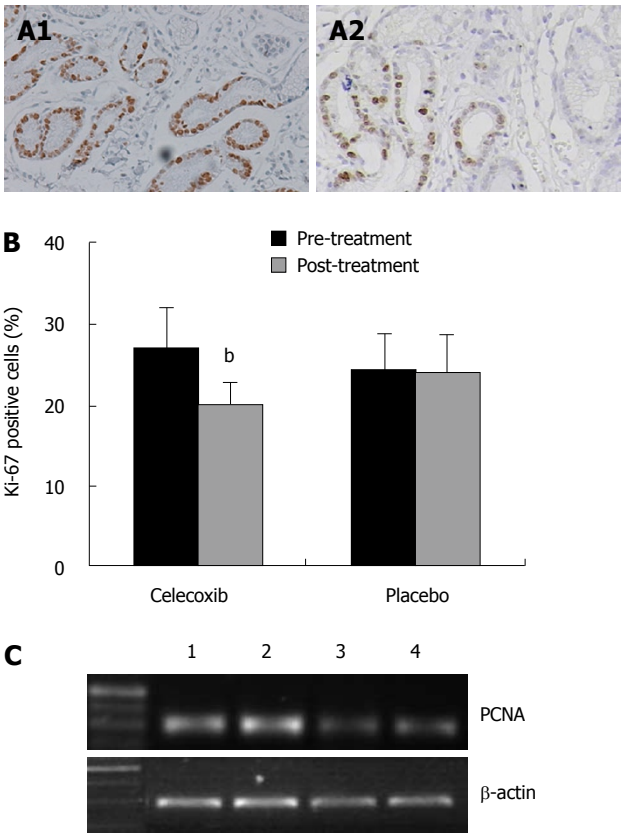

Figure 2 Effects of celecoxib on cell proliferation. A: Representative Ki-67 staining of gastric mucosa before (A1) and after treatment (A2) with celecoxib; B: Celecoxib led to a significant reduction in proliferation index (PI); C: Celecoxib down-regulated mRNA expression of proliferation cell nuclear antigen (PCNA). 1 and 2: Plecebo; 3 and 4: Celecoxib. Data are mean ± SD. $^{b}P < 0.01$.

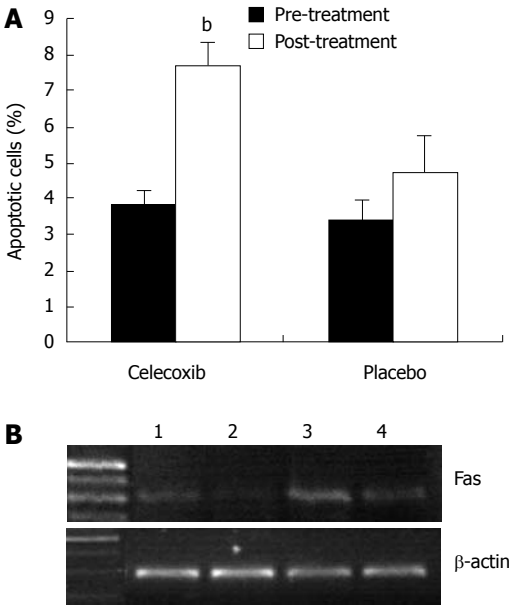

Figure 3 Effects of celecoxib on cell apoptosis. A: Celecoxib induced the apoptosis index (AI) in gastric mucosa; B: Celecoxib up-regulated mRNA expression of Fas, a pro-apoptosis gene. 1 and 2: Plecebo; 3 and 4: Celecoxib. Data are mean ± SD. $^{b}P < 0.01$.

group had reduced PCNA protein expression while a reduction in PCNA expression was observed in only 37% of patients in the placebo group (Table 3). In addition, mRNA expression of PCNA was markedly down-regulated by celecoxib treatment (Figure 2C).

Effect of celecoxib on cell apoptosis

Treatment with celecoxib significantly induced cell apoptosis as assayed by TUNEL staining (pre-treatment: 3.86% ± 0.44% *vs* after-treatment: 7.72% ± 0.64%, $P < 0.01$) (Figure 3A, Table 3). In keeping with this, mRNA expression of the pro-apoptotic gene *fas* was also enhanced (Figure 3B), suggesting that celecoxib-induced apoptosis was *via* up-regulation of *fas* expression.

Effect of celecoxib on angiogenesis

Angiogenesis was evaluated by the MVD assay using CD31 staining. MVD was significantly lower after celecoxib treatment ($P < 0.001$) (Figure 4, Table 3), indicating a suppressive effect on angiogenesis by celecoxib. However, this difference was not seen in the placebo group.

Effect of celecoxib on PPARγ expression

Celecoxib treatment led to an increase in the number of PPARγ positive cells as determined by immunostaining (pre-treatment: 18% ± 4.33% *vs* after-treatment: 22.6% ± 4.3%, $P < 0.05$) (Figure 5, Table 3). Thus, celecoxib resulted in up-regulation of PPARγ expression.

DISCUSSION

We report here that COX-2 is markedly up-regulated in gastric tissues with inflammation and was more prominent during progression in precancerous lesions in *H pylori*-eradicated patients. Moreover, the induction of COX-2 appeared to coincide with increased cell proliferation and angiogenesis. These results suggested that COX-2 was induced by *H pylori* infection and mediated by *H pylori*-associated premalignant gastric lesions. Our observation on the profile of COX-2 expression is supported by previous studies[7,8,13-16],

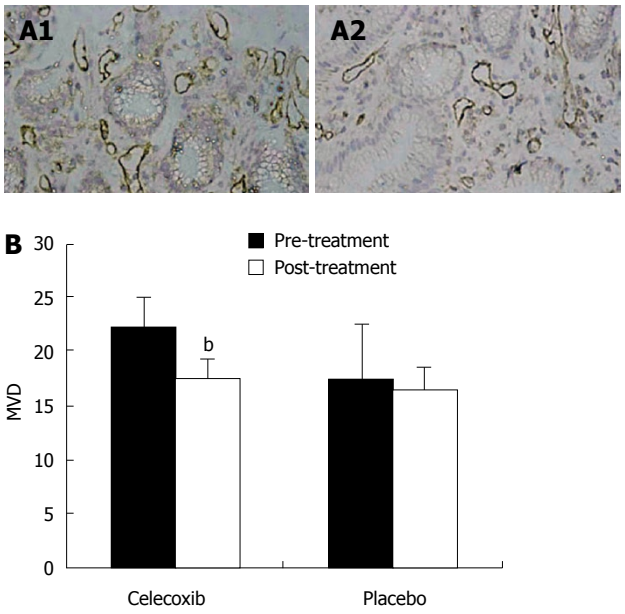

Figure 4 Effects of celecoxib on angiogenesis. A: Representative microvessel image of paraffin-embedded gastric tissue sections stained with CD31. (A1) pre-treatment, and (A2) post-treatment with celecoxib; B: Celecoxib suppressed microvessel density (MVD). Data are mean ± SD. $^{b}P < 0.001$.

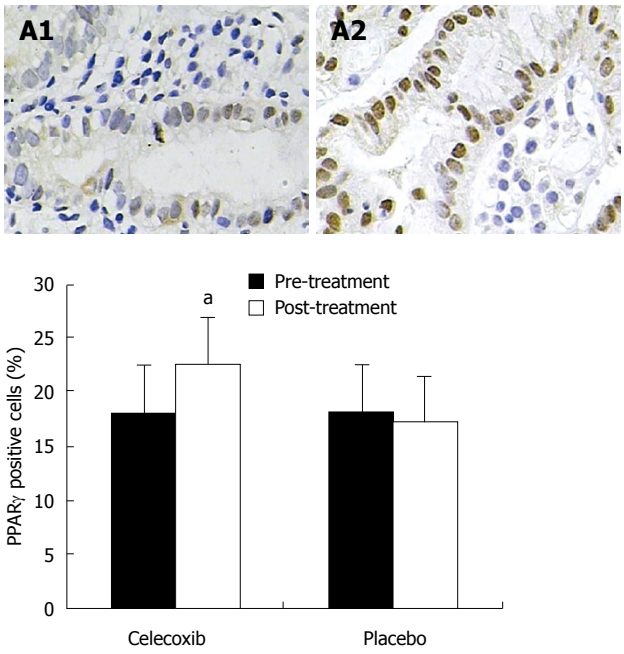

Figure 5 Effects of celecoxib on PPARγ protein expression. Representative PPARγ expression in paraffin-embedded gastric tissue sections by immunostaining. (A1) pre-treatment, and (A2) post-treatment with celecoxib. Nuclear staining of PPARγ was markedly increased post-treatment with celecoxib. $^{a}P < 0.05$.

suggesting that COX-2 is a relatively early event and plays an important role during gastric carcinogenesis.

To evaluate whether progression of precancerous lesions could be reduced or reversed, we conducted a 3-mo intervention of celecoxib in patients with precancerous lesions after *H pylori* eradication. Our results showed that the histology of precancerous lesions was improved in 66.7% of patients treated with celecoxib, which was significantly higher than the placebo group (16.1%) ($P < 0.001$). Of the three precancerous changes (atrophy, intestinal metaplasia and dysplasia), celecoxib was effective on the regression of dysplasia. However, the evidence for chemopreventive effects on gastric precancerous lesions by NSAIDs has only been limited to animal experiments[13,17-22]. In the animal model of carcinogenesis induced by co-treatment with *H pylori* and N-methyl-N-nitrosourea (MNU), mice underwent *H pylori*-induced gastritis with multifocal atrophy and intestinal metaplasia, and finally gastric adenocarcinoma. Long term co-administration with a COX-2 inhibitor, either celecoxib or nimesulide not only reduced the development of intestinal metaplasia, but also adenocarcinoma[20-22]. In human studies, a recent report showed that treatment with NSAIDs for more than 3 mo reversed *H pylori*-induced harmful effects in gastric epithelial cells[23]. On the other hand, large-scale clinical trials have shown that NSAIDs were effective in the chemoprevention of colorectal neoplasia[5]. NSAIDs (celecoxib and sulindac) promoted regression in both number and size in high risk individuals with familial adenomatous polyposis[5]. In the more common sporadic setting, refecoxib and celecoxib reduced the occurrence of human colorectal adenomas[5]. Collectively, these results suggest that the protective effects of NSAIDs such as celecoxib could effectively prevent or reverse the precancerous lesions of gastric cancer.

We further evaluated the underlying mechanisms for the anti-tumorigenic effects of the COX-2 inhibitor. On the basis of the cell proliferation and apoptosis analyses, improvements in precancerous lesions caused by celecoxib were most likely associated with the suppression of cell proliferation and induction of cell apoptosis. In keeping with this, in an animal experiment, celecoxib but not indomethacin suppressed gastric cancer formation by inducing cell apoptosis and suppressing cell proliferation[24,25] in a dose dependent manner. A recent report also indicated that NSAIDs induced apoptosis through activation of extrinsic and intrinsic pathways of apoptosis[26]. However, the pro-apoptotic efficacy of various NSAIDs differed greatly[27,28]. That may explain why the anti-tumor effects of NSAIDs varied in different experiments.

In the present work, we showed that, in addition to inhibition of cell proliferation and induction of apoptosis, the regression of precancerous lesions in the stomach by celecoxib was also related to inhibition of angiogenesis. It is well-established that the inducible enzyme COX-2 is an important mediator of angiogenesis during tumor growth[29]. COX-2 expression significantly correlated with MVD[30] and vascular endothelial growth factor (VEGF) in human gastric adenomas and carcinomas[31]. The pro-angiogenic effects of COX-2 are mediated primarily by the metabolites of arachidonic acid, resulting in increased production of VEGF, enhanced survival of endothelial cells, induction of matrix metalloproteinases, promotion of vascular sprouting and migration and activation of epidermal growth factor receptor-mediated angiogenesis[29]. In this regard, we showed in this study that induction of

COX-2 is parallel with the induced angiogenesis in precancerous lesions. Treatment with celecoxib inhibited angiogenesis with a concomitant histological regression of precancerous lesions. Others have also reported that NSAIDs including celecoxib inhibited angiogenesis and decreased tumor growth in gastric cancer and other cancers both *in vitro* and *in vivo* in animal models[13, 32-34]. Our study provided the first clinical evidence that treatment with celecoxib effectively suppressed angiogenesis and lowered MVD in *H pylori*-eradicated patients with gastric precancerous lesions.

The expression of PPARγ, a protective anti-neoplastic molecule[35], was enhanced by the COX-2 inhibitor rofecoxib in human gastric cancer with a concomitant induction of apoptosis and attenuation of proinflammatory cytokines production[36]. We also observed the up-regulation of PPARγ in patients treated with celecoxib. The up-regulation of PPARγ by NSAIDs was reported either *via* the COX-2 independent pathway or the COX-2 dependent pathway[37-39]. Activation of PPARγ was shown by us and others to prevent mammary carcinogenesis in experimental animals[40-42] through suppression of COX-2 expression[40]. In mice treated with MNU and *H pylori*, nimesulide administration substantially reduced *H pylori*-associated gastric tumorigenesis along with substantial activation of PPARγ and induction of apoptosis[21]. Collectively, these findings raise the possibility that up-regulation of PPARγ by celecoxib contributed to the histological improvement in precancerous lesions.

In conclusion, COX-2 expression was induced in gastric epithelium with the progression of precancerous lesions. Eradication of *H pylori* combined with a 3-mo intervention of celecoxib was effective in improving the severity of precancerous lesions mainly by inducing apoptosis, and inhibiting cell proliferation and angiogenesis. Thus COX-2 is a promising target in reversing gastric precancerous lesions and celecoxib showed efficacy in the chemoprevention of these lesions.

COMMENTS

Background

Epidemiologic studies have shown that cyclooxygenase 2 (COX-2) inhibitor could reduce the risk of gastric cancer. The authors aim to evaluate whether celecoxib, a selective COX-2 inhibitor, could reduce the severity of gastric precancerous lesions following *Helicobacter pylori* (*H pylori*) eradication.

Research frontiers

Gastric cancer is the most common cancer and the leading cause of cancer-related death in China, with an overall 5-year survival rate of only 10%-20%. There is a compelling need to explore the novel targets that contribute to gastric carcinogenesis for effective treatment. The development of gastric cancer is generally believed to be a multi-step progression from chronic gastritis to atrophy, intestinal metaplasia (IM), dysplasia and cancer, that is triggered by *H pylori* infection. Eradication of *H pylori* alone is not efficient in preventing the progression of gastric IM. The authors hypothesized in addition to eradicate *H pylori*, inhibition of COX-2, a potential oncogene gene that was induced in the early stage of gastric carcinogenesis, by selective COX-2 inhibitor (celecoxib) may regress the premalignant changes in the stomach by suppressing COX-2. Herein, they tested the effect of a specific COX-2 inhibitor in patients with confirmed gastric atrophy and/or IM after *H pylori* eradication. The present study was a prospective, randomized, and placebo-controlled study.

Innovations and breakthroughs

The authors have demonstrated in this study that *H pylori* eradication therapy followed by celecoxib treatment improves and dampens the progression of gastric precancerous lesions. The anti-neoplastic properties of celecoxib were due to its ability of inhibiting COX-2 activity, inducing apoptosis, suppressing cell proliferation and angiogenesis.

Applications

COX-2 was a promising target in reversing gastric precancerous lesions and celecoxib showed efficacy in this chemoprevention. This finding may provide clinical implication.

Peer review

The authors report interesting data on the effect of celecoxib administration in patients with gastric precancerous lesions following *H pylori* eradicated. This is a well designed study with interesting results.

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Predictive value of multi-detector computed tomography for accurate diagnosis of serous cystadenoma: Radiologic-pathologic correlation

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Abstract

AIM: To identify multi-detector computed tomography (MDCT) features most predictive of serous cystadenomas (SCAs), correlating with histopathology, and to study the impact of cyst size and MDCT technique on reader performance.

METHODS: The MDCT scans of 164 patients with surgically verified pancreatic cystic lesions were reviewed by two readers to study the predictive value of various morphological features for establishing a diagnosis of SCAs. Accuracy in lesion characterization and reader confidence were correlated with lesion size ($\leqslant$ 3 cm or $\geqslant$ 3 cm) and scanning protocols (dedicated *vs* routine).

RESULTS: 28/164 cysts (mean size, 39 mm; range, 8-92 mm) were diagnosed as SCA on pathology. The MDCT features predictive of diagnosis of SCA were microcystic appearance (22/28, 78.6%), surface lobulations (25/28, 89.3%) and central scar (9/28, 32.4%). Stepwise logistic regression analysis showed that only microcystic appearance was significant for CT diagnosis of SCA (P = 0.0001). The sensitivity, specificity and PPV of central scar and of combined microcystic appearance and lobulations were 32.4%/100%/100% and 68%/100%/100%, respectively. The reader confidence was higher for lesions > 3 cm (P = 0.02) and for MDCT scans performed using thin collimation (1.25-2.5 mm) compared to routine 5 mm collimation exams (P > 0.05).

CONCLUSION: Central scar on MDCT is diagnostic of SCA but is seen in only one third of SCAs. Microcystic morphology is the most significant CT feature in diagnosis of SCA. A combination of microcystic appearance and surface lobulations offers accuracy comparable to central scar with higher sensitivity.

Key words: Pancreas; Serous cystadenoma; Multi-detector computed tomography; Central scar; Lobulations; Microcystic

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INTRODUCTION

Pancreatic serous cystadenomas (SCAs) are rare lesions that are almost always benign and usually asymptomatic[1]. SCAs comprise 1%-2% of pancreatic neoplasms and 10%-15% of cystic lesions[2]. While SCAs are relatively uncommon in comparison to pseudocysts and solid tumors of the pancreas, their clinical importance is

indisputable. Though generally regarded as benign, 3% of SCAs have malignant potential with local infiltration and distant metastases[1,3-5]. These are slow growing tumors; however the growth rate varies depending on tumor size. When the tumor is under 4 cm in diameter, the growth rate is only 0.12 cm/year whereas when the tumors ⩾ 4 cm in diameter they can grow at a remarkable 1.98 cm/year[6]. Due to the benign nature and the morbidity associated with pancreatic surgery, a follow up imaging for surveillance is recommended for these tumors[3,7]. Complete resection is considered curative, and is recommended when the lesion is symptomatic, when it increases in size upon follow-up, and when confident non-invasive differentiation from a more aggressive lesion is impossible[6]. Tumors that are resected have a good prognosis, with no requirement for postoperative surveillance[3,6].

Due to increased utilization of high-resolution imaging such as multi-detector computed tomography (MDCT), magnetic resonance imaging (MRI) and MR cholangiopancreatography (MRCP), SCAs are now more frequently identified, and often incidentally[8,9]. This high rate of detection of incidental pancreatic cystic lesions has generated great interest regarding the appropriate management of these lesions[6]. However, there is some overlap in imaging appearance among cystic pancreatic lesions, and it can be difficult to differentiate SCAs from other types of pancreatic cysts, such as pseudocysts, mucinous cystic neoplasms (MCNs) and intraductal papillary mucinous neoplasms (IPMNs). Thus the diagnosis of serous cystadenomas assumes particular significance because they need to be differentiated from other cystic neoplasms like MCNs, which are known to be premalignant or malignant[10]. The differentiation is vital to avoid unnecessary pancreatic surgery, which although increasingly safe in experienced hands continues to cause significant postoperative morbidity[6]. Thus accurate preoperative lesion characterization is crucial in determining appropriate action.

MDCT is the initial imaging modality of choice for evaluation of cystic lesions of the pancreas. Although the imaging features of SCAs on MDCT have been described before, few studies have compared the accuracy of various CT features for distinguishing SCAs from other lesions. With this purpose in mind, we undertook this study to examine the different features of SCAs on MDCT and identify the common and uncommon imaging features of SCAs that enable a confident diagnosis. Other commonly occurring cystic lesions were also studied from a large cohort of cystic lesions to identify the specific features that characterize SCAs.

MATERIALS AND METHODS

Study design

This is a retrospective study that was approved by the Institutional Review Board and follows the Health Insurance Portability and Accountability Act regulations. Informed patient consent forms were waived. We searched the electronic database of hospital medical records for patients who had pancreatic surgery for cystic lesions from January 1999 to August 2007. The inclusion criteria required the patients to have had the MDCT exam prior to surgical resection. Out of a total of 180 patients with MDCT studies prior to surgical resection, 16 patients who had clinical and laboratory evidence of acute pancreatitis were excluded from the study. Criteria for diagnosis of pancreatitis were elevated serum amylase or lipase levels and/or imaging evidence of pancreatic inflammation. The process resulted in an initial cohort of 164 patients. These patients were evaluated for clinical presentation, imaging features, and pathological and surgical findings. Of the group of 164 patients, a subset consisting of 28 patients (17 women, 11 men; age range, 29-90 years; mean age, 62 years) with pathologically verified SCAs were studied to analyze their characteristic imaging features and constituted the main population for the present study. However, data from the other 136 patients in the cohort of 164 was also analyzed to study the accuracy of each imaging feature studied.

MDCT technique

All patients had undergone CT examinations on MDCT (GE Health Care, Milwaukee, WI) with four, eight or 16 detector rows. The acquisition protocol for the CT exam was either dedicated pancreatic protocol CT (91/164, 14 SCAs) or a routine contrast enhanced CT study (73/164, 14 SCAs). The initial scan was a non-enhanced CT acquisition of 5 mm thickness through the liver and pancreas. For routine abdominal CT scanning, 120-150 mL of nonionic contrast material (300-370 mg/mL) was injected at a rate of 3.0 mL/s, and 5 mm thick images were acquired after a 65-70 s delay.

Pancreatic protocol consisted of two phase acquisition after administration of 120-150 mL of nonionic contrast material (370 mg of iodine per milliliter) at a rate of 4-5 mL/s. Pancreatic phase imaging was performed 45 s after starting contrast material injection by obtaining 1.25-mm/2.5-mm targeted reconstructed sections through the pancreas. Portal venous phase imaging followed at 65-70 s after contrast material injection with 5-mm-thick sections. For pancreatic phase imaging, the field of view was 28 cm; for the other phases, the field of view was adjusted according to the size of the patient.

Post processing

Coronal reformatted images of 2.5-3 mm thickness were obtained in all of the patients. Additional reformatting techniques used included oblique coronal multiplanar reformations (MPRs, 5 mm) parallel to the pancreatic head or tail, 1-2 curved planar reformations along the course of the pancreatic duct (1.25 mm) and thin slab (5 mm) coronal maximum intensity projections (MIP) to display vessel involvement. These reformations were performed by one of the trained technologists on a work station (ADW 4.0, GE).

Data analysis

The CT images were retrospectively reviewed by consensus by two radiologists with 14 and 7 years of experience, respectively, who were kept blinded to patients' clinical details and histopathology of the cystic lesion. The analysis was done on picture archiving and communication system (PACS version 4.0, Agfa, Richmond, VA). For the image analysis, a template was created to evaluate features of a pancreatic cyst. The following features were assessed: cyst size, presence or absence of septations, lesion margins, solid components, lobulations, central scar, calcification, pancreatic duct communication, duct obstruction, lymphadenopathy and vascular involvement. The pancreas was evaluated for presence or absence of duct dilatation, parenchymal/ductal calcification and parenchymal atrophy.

The largest dimensions of the cyst were measured on axial scans and mean size calculated. The septations were evaluated for thickness and enhancement. The margins of the lesion were considered either well defined or ill defined. The shape of the cysts was defined as either smooth, simple lobulated or complex lobulated. Simple lobulation was defined as the shape of a simple closed curve with bosselated surface whose borders could not be described within the same circle[10]. A complex lobulated shape was defined as one containing a conglomeration of two or more cysts either round, oval or tubular (pleomorphic in shape)[10]. Central scar was defined as a central stellate area of soft tissue density with or without calcification and with or without radiating surrounding linear strands. Attenuation values were obtained for the cysts by placing a ROI (mean size, 20 mm^2) on the unenhanced scan. Care was taken to exclude septations, calcifications and solid component within the ROI. The attenuation values measured from the various types of lesions (SCA, MCN, *etc*) were then averaged for comparison. Cyst morphology was classified as unilocular, microcystic, macrocystic or oligocystic and cyst with solid component[8]. Simple unilocular cysts included pancreatic cysts without internal septa, a solid component or central-cyst wall calcification. Unilocular cysts with mural enhancement or calcification were categorized as complicated. Microcystic lesion was defined as consisting of collection of cysts (> 6) ranging in size from a few millimeters up to 2 cm in size[8]. Macrocystic or oligocystic lesions were defined as lesions with more than one but < 6 cysts with at least one of them > 2 cm in size.

Using these features, the cysts were categorized as a simple cyst/pseudocyst, SCA, mucinous cystadenoma (MCN) and intraductal papillary mucinous cystadenoma (IPMN) or else solid neoplasms with cystic degeneration (Adenocarcinoma, endocrine tumors and solid papillary epithelial neoplasm) on imaging. The criteria considered for diagnosis of SCAs included: lobulations, microcystic pattern, presence or absence of central scar, well defined margins and lack of enhancement. Readers' diagnostic confidence for the diagnosis of serous cystadenoma was rated on a 5-point scale (with 1 being least confident and 5 being most) along with confidence as to whether the lesion was benign or malignant using a similar 5-point scale. Cystic lesions which did not have any specific attributable features to the above categories or those with a reader confidence less than three were considered indeterminate.

Surgery and histopathologic correlation

The type of surgical procedure performed was recorded. Histopathological analysis had been performed by a single gastrointestinal pathologist with ten years of specialized experience in pancreatic pathology. The gross and microscopic descriptions of the resected specimens described in the pathology reports were reviewed. A predefined template for pathology was filled in. The gross specimens were reviewed for cyst morphology, which included lesion size, septations and intralesional solid components. The final histopathological diagnosis was recorded as: SCA, IPMN, MCN, adenocarcinoma, endocrine tumors or solid papillary epithelial neoplasm. Those cysts without specific identifiable features where a conclusive diagnosis was not rendered on histopathology were categorized as unclassified cysts. The features on MDCT were then correlated with pathological reports.

Statistical analysis

Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy for the various MDCT findings of SCA were calculated in comparison to other cystic lesions by using histopathology as the gold standard. *T*-test was used to calculate the statistical significance for various values and $P < 0.05$ was considered to suggest statistically significant difference. Statistical significance between the size of SCA and the occurrence of scar was evaluated using two sided Fisher's exact test. Stepwise logistic regression was used to identify the significance of each CT feature for diagnosis of SCA using SAS software (SAS system release 8.2). The CT feature was considered significant for the diagnosis whenever a tail probability of $P < 0.05$ was reached.

RESULTS

CT findings

Of the 28 SCA (mean size, 39 mm; range, 8-92 mm), 11 lesions were located in the head, 11 in the body, 3 in the tail and 3 in body and tail (Table 1). 22/28 (78.6%) SCAs were observed to have microcystic morphology (Figure 1) and 6/28 (21.4%) of SCAs had macrocystic or oligocystic morphology (Figure 2). 25/28 (89.3%) of SCAs were lobulated whereas 3/28 (10.7%) presented with smooth wall. 9/28 (32.1%) SCAs showed central scar with calcification seen in 3 scars (Figure 3). 8/9 (88.9%) of SCAs with central scar measured at least 2 cm (mean size, 4.1 cm; range, 1.9-8.4 cm). This finding was statistically significant for lesion size and occurrence of central scar ($P = 0.02$, Fisher's exact test).

Of the 136 other cystic lesions included for comparison of the morphological features with SCAs,

Table 1 MDCT findings observed in the various pancreatic cystic lesions

	SCA (*n* = 28)	IPMN (*n* = 42)	MCN (*n* = 37)	Unclassified Cysts (*n* = 45)	Endocrine tumors (*n* = 6)	Adenocarcinoma (*n* = 4)	Lymphan-gioma (*n* = 1)	SPE (*n* = 1)
Size (cm) mean (range)	3.9 (0.8-9.2)	2.7 (0.9-5.2)	4.3 (2.2-11)	2.4 (0.5-7.4)	4.6 (1.6-8.9)	3 (1.4-5.2)	2.3	2.3
Location								
Head	11	26	12	20	2	2	1	
Body	11	7	7	8	0	1		1
Tail	3	7	17	12	4	1		
Body/Tail	3	3	1	5	0	0		
Lobulations (*n* = 35/164)	25	3	1	5	1	0	0	0
Microcystic (*n* = 25/164)	22	1	2	0	0	0	0	0
Central Scar (*n* = 9/164)	9[2]	0	0	0	0	0	0	0
L + M[1] (*n* = 19/164)	19	0	0	0	0	0	0	0
Septa	27	32	25	28	2	2	0	0
Wall								
Thin	28	40	30	31	0	2	1	1
Thick	0	2	7	14	6	2	0	0
Calcifications								
Wall	0	4	1	5	0	0	0	0
Septal	0	2	0	0	0	0	0	0
Parenchymal	1	0	0	0	0	0	0	0
Mural nodules	0	3	6	1	3	4	0	1
Dilatation of PD	3	14	11	2	0	3	0	0
Vascular involvement	0	0	1	2	0	2	0	0
Lymphadenopathy	0	3	6	3	0	2	0	0

MDCT: Multi-detector computed tomography; SCA: Serous cystadenoma; IPMN: Intraductal papillary mucinous neoplasm; MCN: Mucinous cystic neoplasm. [1]Combined presence of Lobulations and microcystic appearance; [2]3/9 showed calcifications within the central scar.

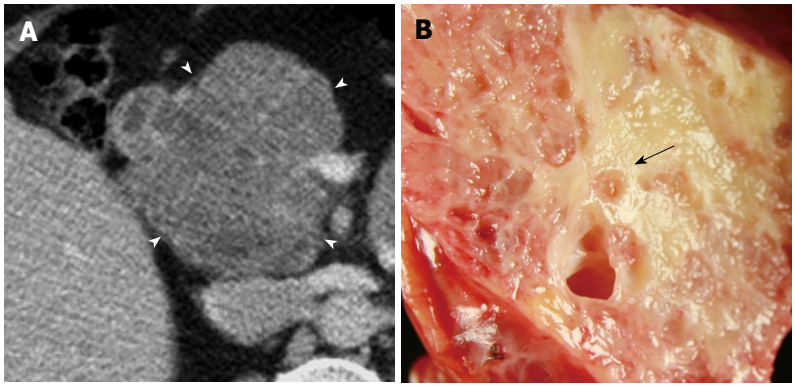

Figure 1 A 64-year-old woman who was incidentally found to have a mass in the head of the pancreas and subsequently underwent a Whipple procedure for removal. A: Lobulated (arrow heads) microcystic serous cystadenoma (SCA) with characteristic honeycomb appearance is seen on axial MDCT image (1.25 mm); B: Gross pathological specimen of the lesion reveals cluster of microcysts with a sponge pattern. A central scar is appreciated on the pathological image (black arrow), which is subtle and difficult to appreciate on the CT image.

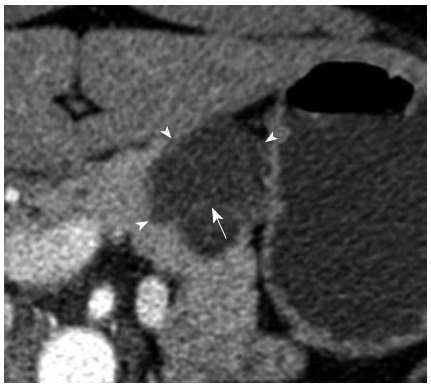

Figure 2 A 46-year-old woman who presented with abdominal pain was found to have a lesion in the body of the pancreas for which a partial pancreatectomy was performed. Axial MDCT image (1.25 mm) shows a lobulated (arrow heads) macrocystic SCA with septations (white arrow).

42 were IPMNs (mean size, 2.7 cm; range 0.9-5.2 cm), 37 were MCNs (mean size, 4.3; range, 2.2-11 cm), 45 were unclassified cysts (mean size, 2.4 cm; range 0.5-7.4 cm), 6 endocrine tumors (mean size, 4.6 cm; range, 1.6-8.9 cm), 4 adenocarcinomas (mean size, 3 cm; range, 1.4-5.2 cm), 1 was lymphangioma (2.3 cm) and 1 was solid papillary epithelial neoplasm (2.3 cm). Among these lesions microcystic pattern on CT was observed in 1 IPMN (2.3%) and 2 MCNs (5.4%) whereas the lobulated pattern was observed in 5 unclassified cysts (11.1%), 3 IPMNs (7.7%) and 1 MCN (3%). Central scar was not demonstrable in any of these lesions.

Upstream pancreatic ductal dilatation was observed in 3 patients. In one patient this was attributed to mass effect from a large SCA in the head of pancreas (Figure 4). However in two other patients with SCA,

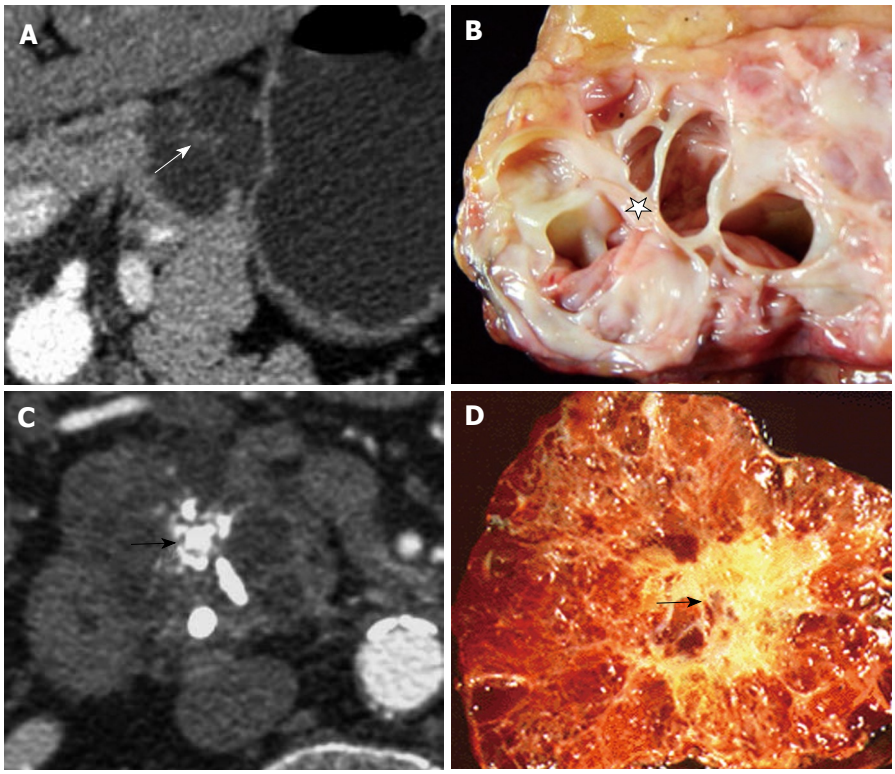

Figure 3 Images from 2 different patients with central scar. A: Axial MDCT image from a 46-year-old woman with a macrocystic SCA with a central scar (white arrow); B: Gross pathological specimen of the same patient also reveals the macrocystic pattern with the septa converging on a central scar (star); C: Axial MDCT image from a 66-year-old male who had a history of chronic pancreatitis, reveals a large lobulated microcystic lesion with central stellate calcification (black arrow). A Whipple procedure was done to remove a 4 cm mass from the head of the pancreas. Corresponding gross pathological image of the microcystic SCA with calcification (black arrow) in the central scar (D).

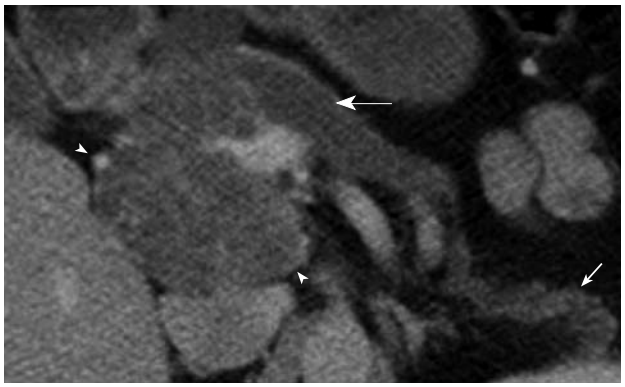

Figure 4 Axial MDCT image from a 64-year-old woman who had a large cyst in the pancreas detected incidentally. The patient underwent a Whipple procedure for removal of the large microcystic SCA (arrow heads) involving the head and uncinate process of the pancreas. The SCA caused upstream pancreatic duct dilatation (white arrow) and atrophy of the pancreatic body and tail (small white arrow).

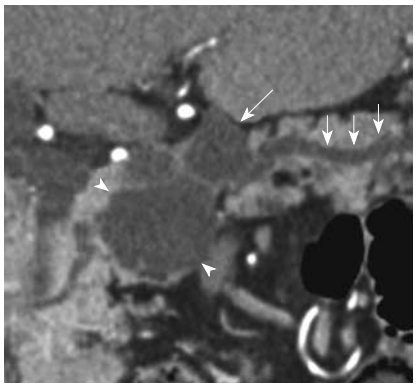

Figure 5 Coronal reformatted MDCT image from an asymptomatic 90-year-old woman, who had a 5 cm lesion in the head of the pancreas which was removed by Whipple procedure. The image shows an oligocystic SCA (arrow heads) in the head of pancreas which was mistaken for a mucinous lesion due to an associated side branch IPMN adjacent to it (white arrow). The two lesions were interpreted as a single multiloculated side branch mucinous lesion. There is mild upstream dilatation of the visualized pancreatic duct (small white arrows).

the pancreatic duct dilatation was considered due to background chronic pancreatitis changes in one and concurrent combined IPMN in another (Figure 5). Furthermore, five patients showed atrophy of the pancreatic parenchyma distal to the site of the lesion (Figures 4 and 6). None of the SCAs showed solid component, ductal communication or vascular encasement. The mean HU values for SCA were 19 ± 9 HU which was higher for microcystic variants (21 ± 8 HU) than the macrocystic variants (13 ± 4 HU). Though the average attenuation value for SCAs was higher than other types of cysts [mucinous lesions (MCNs and IPMNS): 10 ± 6 HU, pseudocysts: 12 ± 4 HU] it was not statistically significant ($P > 0.05$).

CT and histopathological correlation

Twenty four (85.7%) of the 28 SCAs were correctly characterized as such by CT. Of the four SCAs incorrectly identified on CT, two were classified as MCNs, one lesion as a possible IPMN, and one lesion was considered an indeterminate cystic lesion. The two classified as mucinous lesions had a macrocystic appearance and no central scar. One of these was classified as a benign mucinous lesion based on its macrocystic pattern and lack of central scar on CT. The second patient had two cysts, of which one was classified as mucinous and second as IPMN of side branch variety. However, histopathological analysis

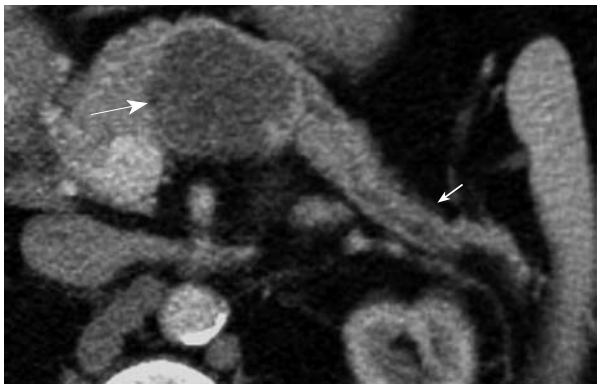

Figure 6 Axial MDCT image from a 64-year-old woman with an incidentally discovered cystic lesion in the body of the pancreas. A large serous cystadenoma (arrow) present in the body of pancreas displays microcystic morphology and fine lobulations with a central scar. Atrophy of the pancreatic parenchyma distal to the site of the lesion is also present (small arrow).

showed that the former was a SCA with a side branch IPMN in the vicinity (Figure 5). The lesion characterized as IPMN was microcystic, but confidence for diagnosis of SCA was low. The lesion classified as indeterminate cystic lesion was lobulated with thin septations and though the morphology was identified as macrocystic and benign, the reader confidence for diagnosis of SCA was less than 3.

When the imaging data of SCA were compared with pathological analysis, the majority of the observations remained constant (Table 2). MDCT features were consistent with the histological data for microcystic appearance (27/28, 96.4%) and surface lobulations (28/28, 100%). The 25 SCAs with pathologically confirmed lobulated morphology and the 3 with pathologically smooth morphology were correctly identified as such by CT. Of the 21 SCAs with pathologically confirmed microcystic morphology and 6 SCAs with pathologically confirmed macrocystic pattern, all were confidently identified as such on CT. One SCA which was characterized as microcystic on CT was found to be unilocular on pathology. This lesion measured 2 cm in diameter and there was substantial background noise on CT images.

Of the 10 central scars discovered on pathology, CT correctly identified them in 8 lesions. Central scars were missed on CT in 2, while in another SCA the central scar recorded on CT was found to represent converging septa on histopathology. In two lesions where CT failed to detect central scar, the SCA measured < 3 cm in size (average, 2.4 cm) and were evaluated using a routine protocol (5 mm). It is conceivable that these factors could have contributed to the reduced accuracy in depiction of SCA morphology.

For cyst characterization into benign and malignant, 27/28 lesions were confidently diagnosed as benign except for one lesion that was considered indeterminate on CT and was found to be benign on histopathology. This lesion measured 5.4 cm in diameter and presented with pancreatic duct dilatation and parenchymal atrophy, which raised the suspicion for an aggressive biology.

Table 2 Comparison of MDCT findings with pathological findings for SCA (n = 28)

	MDCT features	Pathology findings
Morphology		
Microcystic	22	21
Macrocystic/Oligocystic	6	6
Unilocular	-	1
Shape		
Lobulated	25	25
Smooth	3	3
Central scar	9	10
Wall		
Thin	28	28
Thick	0	0
Mural nodules	0	0
Septa	27	27

Morphological features in the diagnosis of SCA

The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy of each of these features in the diagnosis of SCA are shown in Table 3. Based on pathologic verification, microcystic morphology on MDCT was demonstrated in 21/28 (75%) of SCAs. Microcystic pattern was also considered in other cystic lesions on MDCT that included 1 IPMN (n = 42) and 2 MCNs (n = 37), however on histopathology the CT appearance was not corroborated. The presence of microcystic morphology alone thus had a PPV of 88% and a specificity of 97.79% for the diagnosis of SCA. Lobulations were seen in 25/28 (89.3%) of SCAs, 5/45 unclassified cysts (11.1%), 3/42 IPMNs (7.1%) and 1/37 MCNs (2.7%). Lobulations had a PPV of 71.4% and specificity of 92.6% in the diagnosis of SCAs. When both lobulations and microcystic morphology were used for the identification of cystic lesions there was a PPV and specificity of 100% for identification of SCAs. It was found that central scar, microcystic pattern and combined presence of microcystic pattern and lobulations had the highest accuracy in the diagnosis of SCA. Stepwise logistic regression analysis showed that only microcystic appearance was significant for the CT diagnosis of SCA (P = 0.0001).

When comparing the diagnostic confidence for diagnosis of SCAs with reference to the lesion size, the confidence was higher for lesions ⩾ 3 cm (4.6) compared to lesions < 3 cm (3.9, P = 0.02). The CT pathologic concordance was better for lesions scanned with dedicated pancreatic protocol CT. The diagnostic confidence of readers in identifying the morphology of SCAs was better in those lesions scanned with dedicated pancreatic protocol (1.25-2.5 mm) compared to those scanned with routine abdomino-pelvic CT (5 mm) though this was not statistically significant (confidence level, 4.4 *vs* 4, P > 0.05).

DISCUSSION

High rate of incidental detection of cystic pancreatic lesions, including serous cystadenoma and the

Table 3 Sensitivity, specificity, predictive values and accuracy of morphological features in the diagnosis of SCA in comparison to other cystic lesions

	Lobulations (*n* = 35/164, %)	Microcystic (*n* = 25/164, %)	Central scar (*n* = 9/164, %)	L + M[1] (*n* = 19/164, %)
Sensitivity	89.30	78.50	32.40	67.85
Specificity	92.64	97.79	100.00	100.00
Positive predictive value	71.42	88.00	100.00	100.00
Negative Predictive value	97.67	95.70	87.74	93.80
Accuracy	92.00	94.50	88.40	94.50

[1]L + M: Lobulations and microcystic pattern.

overlapping imaging features of SCAs with other more aggressive cystic neoplasms makes management of these lesions challenging[7,9,11,12]. Accurate characterization is therefore imperative because of the low malignant potential of SCA and to determine the treatment options and to differentiate them from more aggressive lesions. Several studies have described the spectrum of imaging appearances of SCA on CT which include microcystic, macrocystic, unilocular and mixed morphologic patterns[7,9,11-14]. Microcystic pattern which is the most predominant pattern has been described in 70%-80% of SCAs[11,12,14]. Surface lobulations considered to be specific for the diagnosis of SCA have been studied with reference to oligocystic SCA (SOAs)[10,15,16]. Central scar which is considered pathognomonic for diagnosis of SCA has a low sensitivity and reliance on this imaging feature will not permit diagnosis of most SCAs[10,11,17]. The accuracy of CT in diagnosis of SCA in comparison to other cystic neoplasms has been reported in several previous studies which found accuracy ranging from 27%-93%[17-19]. More recent studies have compared SOAs with MCNs and IPMNs and have emphasized the importance of patient demographics, lesion location and shape of the cyst in characterization of the various lesions[4,10,15]. However, our study is unique as we have assessed the predictive value of specific features of SCA on MDCT in a large cohort of patients with surgically verified pancreatic cystic lesions and have evaluated how well the CT features correlate with pathology.

In our study, microcystic appearance was the only significant CT feature in the diagnosis of SCA. The combined presence of microcystic appearance and surface lobulations were also the strong predictors of SCA on CT with high PPV and specificity compared to other cystic lesions. Microcystic appearance was evident in 78% of SCAs in our study and this was concordant with other reports[11-14]. However, occasionally other cystic lesions when small in size can also appear microcystic on CT. In our study, small lesion size (< 2 cm) and background image noise potentially contributed to erroneous morphological depiction. Presence of surface lobulations within a cyst is a recognized feature of SCAs and can help to differentiate oligocystic SCAs from MCNs and IPMNs[10,15]. We observed lobulated contour in 89% of SCAs despite different morphologic appearances (microcystic, macrocystic and unilocular), a finding that has also been reported in other studies[10-11,15]. Although uncommon, surface lobulations can be encountered in a few MCNs (2.9%) and IPMNs (7.6%) as also reported by Kim *et al*[10].

The presence of a central scar is considered to be pathognomonic for SCAs, even when there is no distinct microcystic appearance[17]. Finding a central scar was highly specific of SCA in our study. It was not observed in any other cystic lesion but was encountered in only in 32% of SCAs. Other studies have observed it in anywhere from 30% to 45% of microcystic serous adenomas[17,20,21]. Eighty eight point nine percent of SCAs with central scar measured at least 2 cm indicating that larger the lesion size the more likelihood of occurrence of scar. Size of the lesion also determines the detectability of central scar since the two lesions in our study where the central scar was missed measured less than 3 cm in size.

The mean CT attenuation for all SCAs in our data set was higher than the values for other cystic lesions, more so for the microcystic variants though they were not statistically significant. The marginally elevated HU values could be accounted for by the presence of more stroma between the fluid filled sacs and the higher stroma: fluid ratio in SCA than other lesions. However, the attenuation values cannot be used as primary feature for differentiating between various pancreatic cystic lesions.

Lesion size itself can influence the reader confidence as the confidence for diagnosis of SCA was found to be higher for lesions ⩾ 3 cm compared to those with size < 3 cm. In addition to size of the cystic lesions, the MDCT scanning technique also influences the diagnostic accuracy and readers' confidence for lesion characterization.

Although the comparison of MDCT with MRI was not a part of this study, in our experience we have found that MRI might be beneficial when in doubt since the cyst morphology may be better appreciated on MRI. In our study, lesions which underwent dedicated pancreatic protocol examination with thin collimation (1.25-2.5 mm) had an improved reader confidence in the depiction of the morphological features compared to those which were scanned with routine protocol (5 mm). Two lesions where the central scars were missed on CT and the three cases of SCA which were misdiagnosed as mucinous lesions were evaluated by routine protocol, highlighting

the need to perform a dedicated pancreatic protocol CT for superior lesion characterization of cystic pancreatic lesions. We propose that all pancreatic cystic lesions should have at least one pancreatic protocol CT for better lesion characterization which will not only help avoid additional imaging follow up, but also prevent unnecessary surgical interventions.

This study had several limitations. Firstly, only surgically resected tumors were included for evaluation, which introduces a possible selection bias. However, since the majorities of SCAs are benign and are usually not resected, the comparison of MDCT findings with the histopathology findings in surgically resected SCAs adds to the value of the study. Secondly, not all patients had a dedicated pancreatic protocol CT exam. Though this could have affected the evaluation of diagnostic accuracy, it also provided us with an opportunity to study the effect of dedicated pancreatic protocol for evaluation of SCA compared to routine CT.

MDCT allows reliable assessment of the morphological features of SCA as depicted on gross histopathology. Central scar, although pathognomonic for SCA, is uncommonly seen. Microcystic morphology is the most significant CT feature in the diagnosis of SCA. Combined presence of microcystic morphology and surface lobulations offers high accuracy comparable to central scar but with higher sensitivity, thus allowing reliable diagnosis of SCA. Use of a dedicated pancreatic protocol with thin collimation improves the diagnostic accuracy of MDCT and enhances reader confidence.

COMMENTS

Background

Pancreatic serous cystadenomas (SCAs) are rare lesions that are almost always benign and usually asymptomatic. Though surgical resection results in complete cure, follow up surveillance is usually recommended due to their benign nature and the morbidity associated with pancreatic surgery. There is increased incidental detection of pancreatic cysts including SCAs with the use of multi-detector computed tomography (MDCT) and MRI. Accurate characterization of SCAs assumes importance due to their overlapping imaging features with other pancreatic cystic lesions particularly mucinous cystic neoplasms (MCNs) which have a malignant potential. Though several articles have described the imaging features of SCAs, few studies have compared the accuracy of these CT features in distinguishing between SCAs and other lesions. Therefore, this study was undertaken to evaluate the predictive values of various CT features of SCA that allow in its accurate characterization with pathological correlation.

Innovations and breakthroughs

One of the important innovations in the imaging evaluation of pancreatic cysts is the development of MDCT which allows excellent characterization of the morphology of pancreatic cyst. Though several authors have described the CT features of SCA, very few articles have discussed the predictive value of each imaging feature. This articles shows that microcystic appearance is the most significant imaging feature of SCA and the combination of microcystic appearance and surface lobulations is very specific for SCA in comparison to other cystic lesions.

Applications

The most important clinical application of this study is in the diagnosis and characterization of serous cystadenomas when incidentally detected on a CT scan. The finding of superiority of dedicated pancreatic protocol CT over routine protocol helps in the planning of CT examination when encountered with a incidental pancreatic cyst. This article emphasizes the effect of cyst size on lesion characterization and diagnostic accuracy. This calls for further studies for improve characterization of pancreatic cysts by furthering development of high resolution CT and MRI.

Peer review

The important part of the paper is that the study shows that microcystic appearance and lobulated pattern when present together are highly specific for diagnosis of SCA. This study highlights the value of performing a dedicated pancreatic protocol in the evaluation of pancreatic cystic lesions in improving diagnostic accuracy and reader confidence.

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BRIEF ARTICLES

Pre-endoscopic screening for *Helicobacter pylori* and celiac disease in young anemic women

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Abstract

AIM: To evaluate the usefulness of pre-endoscopic serological screening for *Helicobacter pylori* (*H pylori*) infection and celiac disease in women aged < 50 years affected by iron-deficiency anemia (IDA).

METHODS: One hundred and fifteen women aged < 50 years with IDA were tested by human recombinant tissue transglutaminase IgA antibodies (tTG) and anti-*H pylori* IgG antibodies. tTG and *H pylori* IgG antibody were assessed using an enzyme-linked immunosorbent assay (ELISA). All women were invited to undergo upper GI endoscopy. During gastroscopy, biopsies were collected from antrum (n = 3), gastric body (n = 3) and duodenum (n = 4) in all patients, irrespective of test results. The assessment of gastritis was performed according to the Sydney system and celiac disease was classified by Marsh's System.

RESULTS: 45.2% women were test-positive: 41 patients positive for *H pylori* antibodies, 9 patients for tTG and 2 patients for both. The gastroscopy compliance rate of test-positive women was significantly increased with respect to those test-negative (65.4% *vs* 42.8%; Fisher test P = 0.0239). The serological results were confirmed by gastroscopy in 100% of those with positive *H pylori* antibodies, in 50% of those with positive tTG and in 81.5% of test-negative patient. Sensitivity and specificity were 84.8% and 100%, respectively for *H pylori* infection and, 80% and 92.8% for tTG. Twenty-eight patients had positive *H pylori* antibodies and in all the patients, an active *H pylori* infection was found. In particular, in 23 out of 28 (82%) patients with positive *H pylori* antibodies, a likely cause of IDA was found because of the active inflammation involving the gastric body.

CONCLUSION: Anti-*H pylori* IgG antibody and tTG IgA antibody testing is able to select women with IDA to submit for gastroscopy to identify *H pylori* pangastritis and/or celiac disease, likely causes of IDA.

Key words: Iron deficiency anemia; Women; Celiac disease; *Helicobacter pylori* gastritis

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INTRODUCTION

Iron-deficiency anemia (IDA) is common in women aged < 50 years with a prevalence of almost 5% in Western countries[1]. In this population, the balance of iron is often precarious due to menses, pregnancy and breastfeeding and an excessive menstrual flow is experienced by about 30% of women of reproductive age[2]. Menorrhagia is often considered the only cause of iron deficiency anemia, but some studies have shown the usefulness of a gastrointestinal (GI) tract

evaluation by endoscopy, thus indicating a role of the upper and/or lower GI tract as a likely cause of IDA[3-4]. The vast majority of IDA GI causes affect the upper GI tract and, in particular, there is a high prevalence of conditions associated with iron malabsorption such as *Helicobacter pylori* (*H pylori*) related-pangastritis, celiac disease and atrophic body gastritis in IDA premenopausal women[5-6]. On the contrary, as already reported in previous studies, bleeding lesions are infrequent in these patients and in particular in women aged < 50 years[5-8].

The diagnostic workflow in young women affected by IDA is not clearly established. The British Society of Gastroenterology recommends gastroscopy only in IDA women younger than 45 years presenting with GI symptoms[9]. However, the major issue of GI evaluation is that symptoms are often mild and aspecific in IDA women and that gastroscopy is an invasive procedure associated with a high number of refusals[10]. Furthermore, in our previous work on IDA premenopausal women, gastroscopy was performed as part of the diagnostic protocol in all patients, but was deemed unnecessary in almost 30% of the studied women because they were affected only by menorrhagia[5].

As shown in a previous study, non-invasive tests might be helpful in the selection of IDA women having a high probability of being affected by iron malabsorption GI diseases, in order to better address endoscopy and to increase the patients' compliance to the procedure[11].

The aim of the present study was to prospectively evaluate the usefulness of a pre-endoscopic serological screening for *H pylori* infection and celiac disease with the use of two tests (human recombinant tissue transglutaminase IgA antibodies and anti-*H pylori* IgG antibodies) in women aged < 50 affected by IDA in order to increase the compliance for gastroscopy.

MATERIALS AND METHODS

Patients

Between January and July 2006, 400 consecutive women (median age; 38 years) aged < 50 years with iron deficiency anemia were referred to the "Centro delle Microcitemie" of Rome, a public health institution specialized in the diagnosis of thalassemia. In these women the presence of an anemia emerged because they had previously undergone a complete blood count due to fatigue and/or for routine check-up, and were thus referred by their primary care physicians (60%), gynecologists (25.4%), hematologists (7.3%) or other physicians (7.3%) to the above mentioned center in order to exclude alpha- or beta-thalassemia minor, a frequent genetic disorder in the Italian population. In the "Centro delle Microcitemie", a complete blood count, serum iron and ferritin were repeated in all patients, and after the exclusion of α- and β-thalassemia, the presence of IDA was definitely diagnosed. IDA was defined as hemoglobin (Hb) < 12 g/dL with serum ferritin ≤ 20 μg/dL.

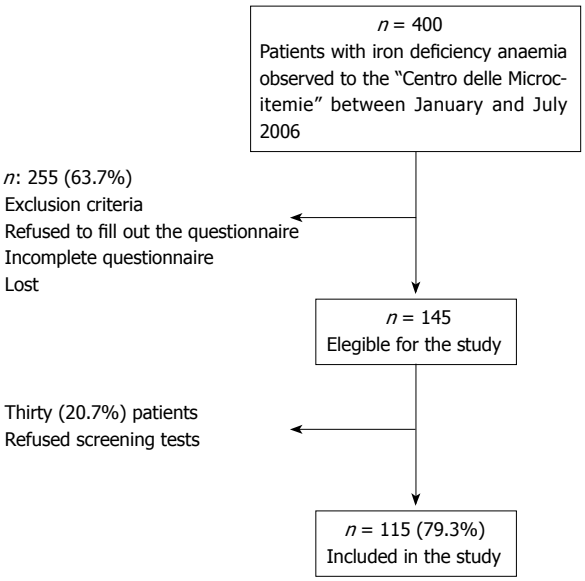

Figure 1 Study population. *n*: Numbers of patients.

IDA women were invited to answer a structured questionnaire to assess demographic data, previous iron supplementation, previous blood transfusion, previous hospitalization for anemia, obvious causes of blood loss, use of drugs (such as aspirin/NSAIDs, proton pump inhibitors, anticoagulants), 1st degree family history for colon or gastric cancer, peptic ulcer and celiac disease, type of diet, premenopausal status and GI symptoms. The duration of anemia was expressed as length of time from first diagnosis of IDA (mo) based on medical records. Upper GI symptoms included nausea, vomiting, dysphagia, heartburn, dyspepsia and upper abdominal pain. Lower GI symptoms included diarrhea, constipation, lower abdominal pain and hematochezia. The symptom was considered "present" if the patient referred to its presence at least once a week during the last three months[10]. Premenopausal status was defined as the patients' personal reports that within 3 mo prior to evaluation they were still menstruating[5].

The women were excluded from the study if they had: age > 50, obvious causes of GI bleeding, previous diagnosis of GI diseases probably responsible for IDA based on medical records, anorexia, vegetarian diet, pregnancy, breastfeeding, anemia of chronic diseases (for example chronic renal failure, cirrhosis and severe cardiopulmonary disease) and hematological diagnoses (e.g. aplastic anemia, myelodysplasia).

Study design

As shown in Figure 1, out of the initial 400 women with IDA, 145 women were considered eligible for the study (Figure 1). The diagnostic work-up included serological tests for the testing of anti-*H pylori* IgG antibodies to evaluate the presence of *H pylori* infection and tissue transglutaminase IgA antibodies to diagnose celiac disease. However, 30 women refused screening tests and thus, 115 women were included in the study and gave their informed consent.

Thus, 115 women were referred to University

Gastroenterology Department to pick up test results and were invited to undergo upper GI endoscopy to confirm test results. Patients with at least one of the 2 tests positive were defined as "test-positive" patients, those with all 2 tests negative were defined as "test-negative" patients.

Measurements

Serologic testing: Human recombinant tissue transglutaminase IgA antibodies were assessed using an enzyme-linked immunosorbent assay (ELISA) based on a commercially available kit (Bio-Rad Laboratories, Milan, Italy). A titre > 15 UI/mL was considered positive. *H pylori* IgG antibodies were assessed using an enzyme-linked immunosorbent assay (ELISA) based on a commercially available kit (Biohit, Helsinki, Finland). A titre > 1.1 UI/mL was considered positive.

Gastroscopy and histological evaluation: During gastroscopy, biopsies were collected from antrum (n = 3), gastric body (n = 3) and duodenum (n = 4) in all patients, irrespective of tests results. The assessment of gastritis was performed according to the Sydney system[12]. *Pangastritis* was defined as the presence of acute and chronic inflammatory infiltrate both in the gastric antrum and body as previously described[13]. If the sum of the inflammatory scores (acute and chronic) showed a two-grade difference between the antrum and corpus, the gastritis was considered as "*antrum-predominant*" or "*corpus-predominant*", respectively. *Antrum-restricted gastritis* was defined by the presence of acute and chronic inflammatory infiltrate exclusively in antrum according to the Sydney system[12]. Celiac disease was classified by Marsh's System[14]. The pathologist was unaware of the serological screening results.

Statistical analysis

Standard descriptive statistics was expressed as median and range and evaluated by appropriate statistical test (Mann-Whitney). Proportions were compared with the Fisher exact test. $P < 0.05$ was considered statistically significant.

RESULTS

Clinical features

The women included in the study had a median age of 38 years (range 21-50 years) and the median duration of IDA was 12 mo (range 1-408 mo). The value of median hemoglobin was 10.7 (range 7.3-11.9) g/dL, MCV was 72 (range 51-92) fL and ferritin was 10 (range 1-20) μg/L. Oral iron therapy was previously prescribed in 48 (41.7%) women. Only a small number of patients had previously needed blood transfusion (n = 3, 2.6%) or hospitalization for anemia (n = 8, 7%).

At least one symptom of the upper GI tract was present in 49.6% of patients (n = 57), while 57.4% of patients (n = 66) had a least one symptom of the lower GI tract. Reported GI symptoms were mainly non-specific, such as mild abdominal pain (24%) and bloating (46%). The vast majority of patients had a negative family history for GI diseases (80.8%): a family history for GI cancers or peptic ulcers was present only in 4.3% and 14% of patients, respectively. In 91.3% of women a premenopausal status was present.

Table 1 Clinical and biochemical features of the "test-positive" patients compared with "test-negative" patients

Patients, n = 115	Test-positive n = 52 (45.2%)	Test-negative n = 63 (54.8%)	P
Age (yr)	38 (23-50)	37.5 (21-50)	NS
Duration of Anemia (mo)	12 (1-408)	12 (1-312)	NS
Hb (g/dL)	10.6 (7.3-11.9)	10.9 (8-11.9)	NS
MCV (fl)	71 (51-90)	74 (58-92)	NS
Iron (μg/dL)	22 (6-65)	24.5 (9-52)	NS
Ferritin (μg/L)	10 (1-31)	10 (2-59)	NS
Smokers	10 (19%)	10 (16%)	NS
Previous therapy with iron	22 (41.5%)	26 (41.9%)	NS
Previous blood transfusion	2 (3.8%)	1 (1.8%)	NS
Previous therapy with vitamin B12	5 (9.4%)	9 (14.5%)	NS
Previous therapy with folic acid	18 (34%)	20 (32.2%)	NS
Hospitalization for anemia	3 (5.7%)	5 (9.4%)	NS
Upper GI symptoms	26 (49%)	31 (50%)	NS
Lower GI symptoms	29 (54.7%)	37 (59.7%)	NS
Premenopausal status	49 (94.2%)	56 (88.9%)	NS

Hb: Hemoglobin; MCV: Median corpuscular volume; GI: Gastrointestinal; NS: Not significant. All values are reported as median (range) unless otherwise indicated.

Serological tests

Fifty-two out of 115 patients (45.2%) were "test-positive". Of these, 41 (35.6%) patients had positivity for *H pylori* IgG antibodies, 9 (7.8%) patients for tissue transglutaminase IgA antibodies and 2 (1.7%) patients had positivity for both antibodies. As shown in Table 1, the group of patients with positive tests was not different from that with negative tests as far as clinical and biochemical features were concerned.

Gastroscopic/histological findings

"Test-Positive Patients": Four women did not undergo gastroscopy because they become pregnant after the start of the study. Of these, 3 patients had positive *H pylori* antibodies and 1 patient positive tissue transglutaminase IgA antibodies. Fourteen patients refused the invasive procedure, 12 out of these patients had positive *H pylori* antibodies and 2 patients had positive tissue transglutaminase IgA antibodies. Thus, 34 out of 52 (65.4%) "test-positive" patients consented to the upper GI endoscopy and the results are shown in Table 2.

Twenty-eight patients had positive *H pylori* antibodies, and in all these patients an active *H pylori* infection was found. Celiac disease was confirmed only in 4 out of 8 (50%) patients with positive tissue transglutaminase IgA antibodies, whereas in another patient who underwent gastroscopy for positive *H pylori* antibodies, celiac disease was also found. Of the 5 patients with celiac disease, 4 had Marsh 3 and 1 had Marsh 2.

In Table 3, the extent and the degree of *H pylori*-related gastritis is shown. Five IDA women with positive *H pylori* antibodies had exclusively *antrum-restricted gastritis*

Table 2 Gastroscopic/histological findings of patients according to diagnostic tests results

Patients	Test-positive $n = 34$			Test-negative $n = 27$
Gastroscopic/histological findings	*H pylori* antibodies $n = 26$	Tissue transglutaminase antibodies $n = 6$	*H pylori* & Tissue transglutaminase antibodies $n = 2$	
H pylori-related gastritis	26	-	2	5
Celiac disease	1	3	1	-
Normal	-	3	-	22
Total findings	27[1]	6	3[1]	27

[1]1 patient had both *H pylori*-related gastritis and celiac disease.

Table 3 Extent and degree of *H pylori*-related gastritis

Histological findings	Test-positive patients	Test-negative patients
Antrum-restricted gastritis	5	2
Pangastritis[1]	21	1
Antrum-predominant pangastritis	1	1
Corpus-predominant pangastritis	1	-
Atrophic body gastritis	-	1
Total	$n = 28$	$n = 5$

[1]Equal score of inflammatory infiltrate in antrum and corpus.

with active *H pylori* infection. In the remaining patients ($n = 23$), a pangastritis with active *H pylori* infection was present which in 91.3% of cases showed equal severity of the inflammatory score in antrum and corpus. Thus, in 23 out of 28 (82%) patients with positive *H pylori* antibodies, a likely cause of IDA was found because the active inflammation involved the gastric body.

"Test-Negative Patients": Three women could not undergo gastroscopy because they were pregnant, and 33 patients refused the procedure. Thus, 27 out of 63 (43%) "test-negative" patients underwent upper GI endoscopy. In 22 out of 27 (81.5%) patients, the negative serological tests results were confirmed because no gastroscopic/histological finding was revealed; instead in the remaining 5 patients *H pylori* gastritis was diagnosed (Table 2). In particular, 3 likely causes of IDA were misdiagnosed because 2 patients had "*antral-predominant*" chronic gastritis and 1 patient had atrophic body gastritis (Table 3).

The compliance rate of test-positive women (65.4%) was significantly higher than that of test-negative ones (42.8%) (Fisher test $P = 0.0239$). Patients undergoing gastroscopy were similar to the group of included patients in the study for demographic, clinical and biochemical data. Moreover, for these parameters, the group of dropped-out test-positive and test-negative patients was not different from those test-positive and test-negative patients who underwent gastroscopy (data not shown).

Diagnostic performance of tissue transglutaminase and anti-H pylori antibodies in IDA

On the basis of these results, the sensitivity, the specificity, and the positive and negative predictive values of tissue transglutaminase IgA antibodies for the diagnosis of celiac disease were 80%, 92.8%, 50% and 98.1%, respectively. The sensitivity, the specificity, and the positive and negative predictive values of anti-*H pylori* antibodies for the diagnosis of *H pylori* infection were 84.8%, 100%, 100% and 84.8%, respectively.

DISCUSSION

In the present study, 115 women aged < 50 years with unexplained IDA were tested by anti-*H pylori* IgG antibodies and human recombinant tissue transglutaminase IgA antibodies to diagnose *H pylori* infection and celiac disease. Almost half of the studied patients tested positive for at least one serological assay, and the suspicion of an upper GI disease as likely cause of IDA, raised by the serological result, was confirmed by gastroscopy in 100% of those with positive *H pylori* antibodies and in 50% of those with positive tissue transglutaminase IgA. On the other hand, in only 11% of test negative patients, gastroscopy with biopsies yielded a finding which may be interpreted as a likely cause of IDA (2 *H pylori*-related *antrum predominant* pangastritis and 1 atrophic body gastritis). Thus, these findings indicate that in women aged < 50 years with IDA, the dual-step approach e.g. serological tests and then invasive procedure, may be considered as a useful tool to optimize the use of gastroscopy, avoiding useless procedures, and to reduce the number of expensive histological examinations.

Previous studies have shown that the presence of GI symptoms or the severity of anemia were related to higher risk of GI causes of IDA[3,5-6]. In this study, no difference was found between test-positive and test-negative patients in terms of personal data, clinical and biochemical features including the frequency of upper GI symptoms and Hb values. Therefore, our results show that GI evaluation is of poor utility to target IDA patients for gastroscopy, strengthening the usefulness of the pre-endoscopic screening by serological tests.

H pylori infection was found in 35.6% of the investigated women, confirming the strong association between *H pylori* infection and IDA observed in previous epidemiological studies[15-16]. Since *H pylori* IgG antibodies do not discriminate between active or previous infection, they are not generally considered useful for diagnosing *H pylori* infection[17]; in this clinical setting of IDA women, *H pylori* antibody-positivity was always associated with active infection as shown by histological data (Table 3). Moreover, 82% of patients with *H pylori* antibody-positivity had gastritis involving the gastric body, while only five cases had an *antrum-restricted* gastritis. In fact, only when the inflammation involves the gastric body, the acid secretion is reduced and the iron absorption is impaired, is there a consequential IDA[13,18]. The presence of positive *H pylori* antibodies, in a patient at high risk for iron malabsorption diseases, supports the need of an accurate gastroscopic/histological evaluation with antral and corporal biopsies to define the extent of gastritis and

eventually its association with IDA. In our clinical setting, we observed 100% specificity and positive predictive value as well as a sensitivity of 85% for the *H pylori* antibodies assay. On the basis of this result, we believe that the assay of *H pylori* antibodies may be considered useful in the selection of IDA women aged < 50 years to submit for gastroscopy, also keeping in mind that this serological test is widely available and cheap and, for this reason, it may be used in the primary care.

Human recombinant tissue transglutaminase IgA antibodies were found to be positive in almost 7% of all patients. This antibody was chosen as screening test for celiac disease, because it is based on ELISA assay and is more accurate compared to the immunofluorescence method used for determining endomysial antibodies[19]. Our results showed a good sensitivity (80%), and specificity (93%) of the assay in keeping with a recent meta-analysis[20]. Our study confirmed also the poor positive predictive value (50%), which is a well known limit of this serological assay. Yet, this value is higher than the one (28%) reported in previous literature[21]. This difference may be explained by the fact that the women included in the present study were anemic and thus were at high risk for celiac disease[22]. Considering the occurrence of false positives of the tissue transglutaminase assay, even in this particular clinical setting, the need for a histological confirmation of celiac disease diagnosis is confirmed.

The main limitation of this work is the small size of the sample, in part due to the high percentage of patients who have refused to participate in the study and of those who, once included, refused gastroscopy. Thus, our findings suggest that the pre-selection of young women for gastroscopy by non-invasive serological testing is able to increase compliance for the invasive procedure. In fact, the compliance rate of test-positive women was significantly increased with respect to those test-negative (65.4% *vs* 42.8%; Fisher test $P = 0.0239$).

In conclusion, half of IDA women aged < 50 years tested positive to serological screening for *H pylori* infection and/or celiac disease, and gastroscopy with biopsies confirmed in the vast majority of them the presence of active *H pylori* gastritis involving gastric body, or celiac disease, as possible causes of IDA. Thus, two simple and widely available tests (tissue transglutaminase IgA antibodies and anti-*H pylori* IgG antibodies) are able to select women with IDA to submit for gastroscopy to identify IDA-related GI causes.

COMMENTS

Background

Iron-deficiency anemia (IDA) is common in women aged < 50 years. Menorrhagia is often considered the only cause of iron deficiency anemia, but some studies have shown the usefulness of a gastrointestinal (GI) tract evaluation. The vast majority of IDA GI causes affect the upper GI tract and there is a high prevalence of conditions associated with iron malabsorption such as *Helicobacter pylori* (*H pylori*) related-pangastritis, celiac disease and atrophic body gastritis in IDA premenopausal women.

Research frontiers

The diagnostic workflow in young women affected by IDA is not clearly established. The British Society of Gastroenterology recommends gastroscopy only in IDA women younger than 45 years presenting with GI symptoms. However, symptoms are often mild and aspecific in IDA women and the gastroscopy is an invasive procedure associated with a high number of refusals. In a previous work on IDA premenopausal women, gastroscopy was performed in all patients, later deemed unnecessary in almost 30% of the studied women because these were affected only by menorrhagia.

Innovations and breakthroughs

This study showed that two simple and widely available tests, ie those for tissue transglutaminase IgA antibodies and anti-*H pylori* IgG antibodies, are able to select women with IDA to submit for gastroscopy to identify IDA-related GI causes and to increase the compliance for the invasive procedure. Gastroscopy with biopsies confirmed in the vast majority of IDA women the presence of active *H pylori* pangastritis, atrophic gastric body, or celiac disease as possible causes of IDA.

Applications

This study showed that a pre-endoscopic serological screening for *H pylori* infection and celiac disease is useful to select IDA women having a high probability of being affected by iron malabsorption GI diseases and to increase the patients' compliance for the procedure.

Terminology

Pangastritis: *H pylori*-related gastritis involving the antrum and the body of the stomach. Malabsorptive diseases: diseases related to iron-malabsorption that include *H pylori*-pangastritis, atrophic body gastritis and celiac diseases.

Peer review

The paper is interesting to study the usefulness of non-invasive test to select women with IDA for gastroscopy. These findings may be not applicable to other countries if the prevalence of celiac disease or *H pylori* infection are low.

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BRIEF ARTICLES

Ephrin A2 receptor targeting does not increase adenoviral pancreatic cancer transduction *in vivo*

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Abstract

AIM: To generate an adenoviral vector specifically targeting the EphA2 receptor (EphA2R) highly expressed on pancreatic cancer cells *in vivo*.

METHODS: YSA, a small peptide ligand that binds the EphA2R with high affinity, was inserted into the HI loop of the adenovirus serotype 5 fiber knob. To further increase the specificity of this vector, binding sites for native adenoviral receptors, the coxsackie and adenovirus receptor (CAR) and integrin, were ablated from the viral capsid. The ablated retargeted adenoviral vector was produced on 293T cells. Specific targeting of this novel adenoviral vector to pancreatic cancer was investigated on established human pancreatic cancer cell lines. Upon demonstrating specific *in vitro* targeting, *in vivo* targeting to subcutaneous growing human pancreatic cancer was tested by intravenous and intraperitoneal administration of the ablated adenoviral vector.

RESULTS: Ablation of native cellular binding sites reduced adenoviral transduction at least 100-fold. Insertion of the YSA peptide in the HI loop restored adenoviral transduction of EphA2R-expressing cells but not of cells lacking this receptor. YSA-mediated transduction was inhibited by addition of synthetic YSA peptide. The transduction specificity of the ablated retargeted vector towards human pancreatic cancer cells was enhanced almost 10-fold *in vitro*. In a subsequent *in vivo* study in a nude (*nu/nu*) mouse model however, no increased adenoviral targeting to subcutaneously growing human pancreas cancer nodules was seen upon injection into the tail vein, nor upon injection into the peritoneum.

CONCLUSION: Targeting the EphA2 receptor increases specificity of adenoviral transduction of human pancreatic cancer cells *in vitro* but fails to enhance pancreatic cancer transduction *in vivo*.

Key words: Pancreatic cancer; Adenoviruses; Ephrin A receptor; Targeting; Genetic transduction

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INTRODUCTION

Pancreatic cancer is a devastating disease with a very poor prognosis[1]. The lack of options for curative treatment for pancreatic cancer and other gastrointestinal malignancies warrants a search for novel targets and novel therapies including gene therapy[2,3]. Adenovirus has been used widely as a gene therapy vector to treat solid tumors. After initial negative results in clinical trials with non-replicating vectors, conditional replicating adenoviral vectors have been tested in clinical trials recently. However, these have also shown disappointing efficacy[4,5]. Poor transduction efficiency and specificity of adenoviral vectors appears to be a major problem.

This seems to be the result of low expression of the primary receptor involved in adenoviral transduction on the tumors, the coxsackie and adenovirus receptor (CAR). Development of targeted vectors to circumvent CAR-mediated entry therefore seem to be required to increase the therapeutic potential of this approach.

Incorporation of ligands that bind to receptors highly expressed on cancer cells in the fiber HI loop enhance adenoviral transduction efficiency[6]. We have shown that incorporation of an RGD peptide improved transduction in pancreatic cancer[7]. Tumor specificity of RGD, however, is limited. Therefore, we decided to introduce the YSA peptide (YSAYPDSVPMMS) in the HI loop (van Geer, in review). YSA is a ligand for the EphA2 receptor (EphA2R) that is highly expressed on pancreatic cancers[8] and other solid tumors[9-11]. Since the YSA peptide has a high affinity for this receptor, it can be used for *in vivo* delivery of agents to tissues and tumors expressing the EphA2R[12]. In addition, binding of YSA activates the EphA2R and induces its internalization which may enhance adenoviral uptake. Adenoviral HI loop insertion of the YSA peptide increased the transduction specificity and efficiency both in human pancreatic tumor cell lines and in pancreatic tumor resection specimens *in vitro* (van Geer, in preparation). *In vivo* however, the presence of the native binding sites in the adenoviral capsid will compromise specific targeting. Even *in vitro*, YSA-mediated entry could only be proven upon inhibition of CAR-mediated entry. As we aim to target pancreas cancer *in vivo* we decided to ablate the native adenoviral binding sites of the YSA-targeted vector.

A highly conserved cluster of amino acids on the adenovirus fiber trimer is involved in CAR binding[13]. Site-directed mutagenesis of amino acids in this region was used to identify mutations that affect CAR binding. Mutations in the AB loop[14,15], the DE loop[16], and the FG loop[17] of the fiber knob all abolished CAR binding *in vitro*.

In addition to CAR, binding to integrins can also mediate adenoviral transduction. The presence of integrins on virtually all normal cells will also limit specific transduction of tumor cells. Removal of the integrin-binding motif RGD from the adenoviral penton base indeed enhances tumor specific targeting of adenoviral vectors[18].

To generate an adenoviral vector that targeted pancreatic cancer *in vivo* we therefore decided to combine HI loop insertion of the YSA peptide with ablation of the binding sites for CAR and integrin. The specificity of this doubly-ablated retargeted vector (Ad/ΔF(FG)Δ P-YSA) was determined *in vitro* and subsequently *in vivo*.

MATERIALS AND METHODS

Materials

Anti-fiber monoclonal 4D2 antibody (NeoMarkers, Fremont, California, USA); anti-EphA2R clone D7 (Sigma, Saint Louis, USA), synthetic peptide YSA (Eurogentec, Seraing, Belgium), Basement Membrane Matrix (BD Biosciences, Bedford, MA); luciferase activity was determined using a commercial kit (Promega) and a Berthold luminometer.

Cells

HEK293 cells, the established PC cell lines Capan-1 and Hs766-T, the mouse pre-adipocyte cell line 3T3-L1 and the mouse hepatoma Hepa 1-6 cell lines were obtained from the American Type Culture Collection Rockville, Maryland; BxPC-3 and MIA PaCa-2 were obtained from Boehringer Ingelheim (Belgium). The pancreatic carcinoma cell lines (p6.3 and p10.5) were obtained from Dr. E Jaffee, Johns Hopkins University School of Medicine, Baltimore, MD, USA. Human umbilical vein endothelial cells (HUVECs, passage 1-3) were isolated as described[19] and cultured in Medium-199 (GIBCO-BRL, Paisley, Scotland), supplemented with 20% (v/v) fetal bovine serum, 50 μg/mL heparin (Sigma, St Louis, MO, USA), 6-25 μg/mL endothelial cell growth supplement (ECGS; Sigma), penicillin (100 IU/mL), streptomycin (100 mg/mL) (GIBCO-BRL). Human fibroblasts (passage < 10) were a gift from the department of Genetic and Metabolic diseases AMC, Amsterdam. Fiber-293 cells expressing adenovirus type 5 fiber protein were used as the packaging cell line as previously described. All cells were cultured in Dulbecco's minimal essential medium (DMEM) with 10% fetal bovine serum (heat inactivated); L-glutamine (2 mmol/L) and penicillin (100 IU/mL), streptomycin (100 mg/mL) all from Cambrex Bio Science, Walkersville. All cell lines were cultured at 37℃ in 10% CO_2 atmosphere.

Plasmids

The E1-, E3-deleted adenovirus vector AdHM43 was used for propagation of integrin- and CAR-binding mutated adenovirus[20]. The RGD-peptide coding sequence at the penton base was changed from MNDHAIRGDTFATRAE to MNDTSRAE and the FG-loop of the fiber was deleted (T489, A490, Y491, T492). The cytomegalovirus (CMV) immediate-early promoter controlled enhanced green fluorescent protein (eGFP) and the CMV-controlled luciferase gene were inserted between the PI-Sce and Ceu-I sites of the pAdHM43 plasmid (van Geer, in review). Insertion of the YSA peptide (YSAPDSVPMMS) into pAdHM43-CMV-GFP and CMV/Luc was performed by digestion with BstB1 and ligation with 2 annealed primers: YSA forward: CGAAGTACAGCGCCTACCCCGACGG-CGTGCCCATGATGT. YSA reverse: CGACATCAT-GGGCACGCTGTCGGGGTAGGCGCTGTACTT. Clones identified by restriction enzyme analysis and PCR were sequenced to exclude mutations.

Virus generation, propagation and analysis

Recombinant ablated adenoviral vectors were generated by transfection of HEK 293 adeno fiber-expressing cells with *Pac* I -linearized Ad-CMV-GFP/Luc-YSA. Normal HEK 293 cells were used for the last propagation round as previously described[20]. Adenovirus was purified and concentrated by performing 2 cesium chloride gradients

and dialyzing against PBS. Glycerol was added to a final concentration of 10% (v/v) and virus preparations were stored at -80℃.

Modification of viral genomes were verified by PCR and sequencing using the following primers: fiber-forward: CAAACGCTGTTGGATTTATG; fiber-reverse: GTGTAAGAGGATGTGGCAAAT; RGD forward: TTGGATGTGGACGCCTAC; RGD reverse: AGGTGTCGCCGCGAATGGC.

The anti-fiber monoclonal 4D2 antibody (NeoMarkers, Fremont, California) was used to confirm proper trimerization of the fiber[21]. The number of viral genomic copies (gc) was determined by qPCR as previously described[22].

EphA2 receptor expression

Expression of EphA2R was studied with Western blotting using a 1:1000 dilution of a monoclonal antibody (anti-EphA2R clone D7, Sigma, Saint Louis, USA) and a peroxidase-conjugated anti IgG secondary antibody (1:2500). Cell lysates were prepared in 25 mmol/L Tris HCl pH 7.6, 150 mmol/L NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS containing a cocktail of protease inhibitors (1:250, Roche). The protein concentration was determined with a BCA assay kit (Sigma). Detection was performed using the Lumi-light plus Western Blotting Substrate Kit (Roche, Mannheim, Germany). For receptor localization studies pancreatic tumor cells were gown on cover slips and were fixated 2 d later and stained for EphA2R using a 1:500 of the monoclonal antibody and a 1:2500 peroxidase-conjugated IgG secondary antibody.

Adenoviral transduction

5.0×10^4 cells were plated in 48-well plates and allowed to adhere overnight at 37℃. The next day virus was added (500 or 1000 VP/cell) in 2% DMEM. Blocking was performed by pre-incubating the cells with blocking agents in PBS for 20 min at room temperature. EphA2R was blocked with the synthetic peptide YSA (Eurogentec, Seraing, Belgium). For the bi-specific antibody experiments, 500 gc of virus were incubated for 60 min with 2.5 μL scFv bi-specific antibody (a kind gift from Dr. VW van Beusechem[23]).

In vivo transduction

1.0×10^7 Capan-1 pancreatic cancer cells were 1:1 diluted with Basement Membrane Matrix (BD Biosciences, Bedford, MA) and injected subcutaneously in both flanks of 6-9 wk old female athymic NMRI *nu/nu* mice (Harlem). When the tumor nodule reached a diameter of 0.4 to 0.7 cm, mice were injected intravenously (i.v.) or intraperitoneally (i.p.) with 1.0×10^{11} gc of Ad-/ΔF(FG)ΔP or Ad-/ΔF(FG)ΔP-YSA in 100 μL PBS. Blood was sampled by orbital puncture at 10 min after *i.v.* injection or at 90 min after *i.p.* injection. Three days after injection, mice were sacrificed and organs were harvested, snap frozen and used to determine luciferase activity according to standard procedures (Promega), using a Berthold luminometer. Luciferase activity was normalized for protein content. Viral DNA in blood was purified as described previously[24] and gc were quantified by qPCR using the primers against the CMV promoter (forward: AATGGGCGGTAGGCGTGTA, reverse AGGCGATCTGACGGTTCACTA). Serum aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT) levels were determined using standard clinical chemistry methods.

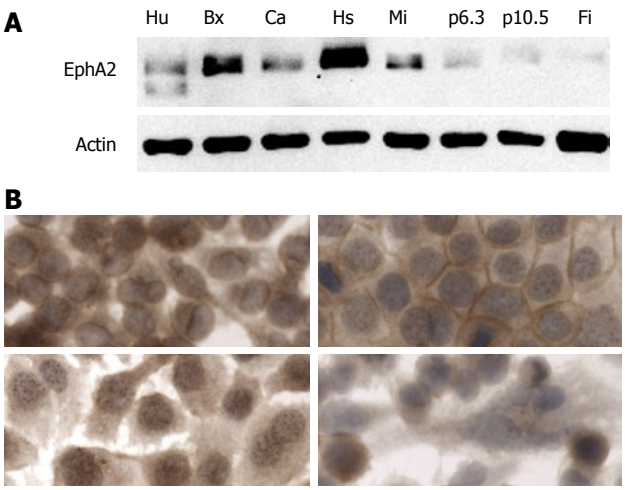

Figure 1 EphA-2 expression in human cell lines. A: Analysis of EphA2R expression by Western blotting in human umbilical vein endothelial cells (Hu), human pancreatic cancer cell lines BxPc-3 (Bx), Hs766-T (Hs), Capan-1 (Ca), MIA PaCa-2 (Mia), p6.3, p10.5 and human fibroblasts (Fi). EphA2R was detected using a monoclonal antibody and detection of actin levels was performed as a loading control; B: Immunolocalization of EphA2R in human pancreatic cancer cell lines BxPc-3 (left top), Capan-1 (right top), Hs766-T (bottom left), MiaPaca-2 (bottom right). Cells were fixated with methanol, acetone, and water, and a directed monoclonal antibody was used to detect EphA2R using a goat anti-mouse labeled with PO to perform DAB detection. Magnification (× 600), except for MiaPaca-2 (× 400).

RESULTS

Expression of the EphA2R on human pancreatic cancer cell lines and tissue specimens

The EphA2R is highly expressed on most solid tumors including pancreatic cancer. To investigate which cell lines are suitable as a model to study targeting, we determined its expression on a panel of established human pancreatic cancer cells and normal human cells such as fibroblasts and endothelial cells. The expression level of the EphA2R varied significantly in human pancreas cancer cell lines (Figure 1A). High expression was seen in Capan1, BxPc3, Hs766T and MIA PaCa-2, while expression in p6.3 and p10.5 was low. Of the normal cells, only human endothelial cells expressed EphA2R. Since this receptor is absent on normal human fibroblasts, we used these as negative control cells.

To investigate whether the EphA2R was accessible, we determined its localization in the cell lines highly expressing this receptor. The localization of this receptor differed between cell lines (Figure 1B). In Capan-1 and BxPc-3 cells, the EphA2R was detected on the membrane while in MIA PaCa-2, it was mostly seen in the cytoplasm. The signal seen in Hs766-T suggests

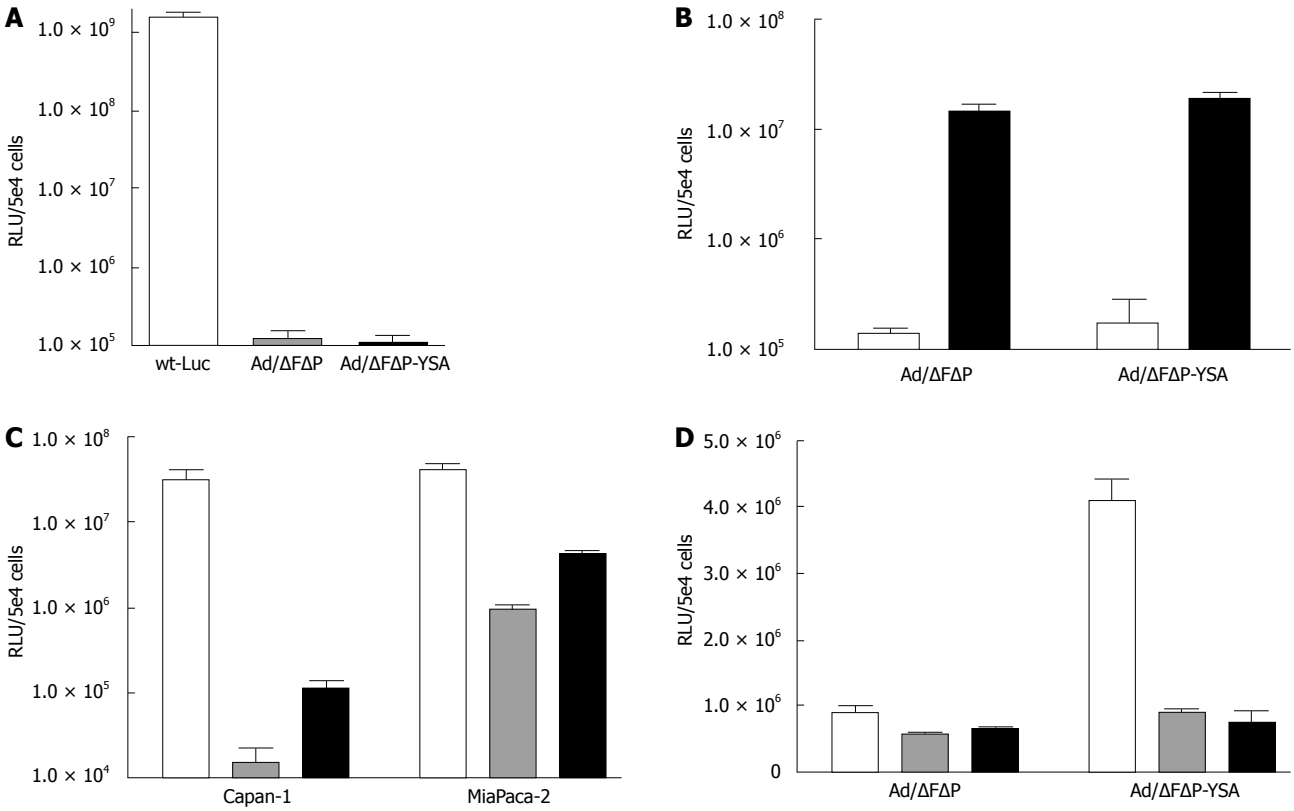

Figure 2 YSA redirects adenovirus to the EphA2 receptor. A: Transduction of CAR-expressing A549 cells with wild-type, ablated and YSA-retargeted adenoviral vectors with 1000 gc/cell: wt-Ad-Luc, Ad-Luc/ΔF(FG)ΔP and Ad-Luc/ΔF(FG)ΔP-YSA. Twenty four hours after infection cells were lysed to determine luciferase levels. Data are expressed as mean ± SD (n = 3). B: Incubation with bi-specific antibody restores infectivity of ablated adenoviral vectors in human fibroblasts. Cells were transduced with 500 gc/cell of Ad-/ΔF(FG)ΔP or Ad-/ΔF(FG)ΔP-YSA with (black bars) or without (white bars) EGF receptor targeted bi-specific scFV molecules. Luciferase activity was measured after 24 h and data are expressed as mean ± SD (n = 3). C: Insertion of YSA peptide in the HI loop of ablated adenoviral vector partially restores transduction of human pancreatic cancer cell lines Capan-1 and MiaPaca-2. Cells were transduced with 1000 gc/cell of wt-Ad-Luc (white bars), or Ad-Luc/ΔF(FG)ΔP (gray bars) or Ad-Luc/ΔF(FG)ΔP-YSA (black bars). Luciferase activity was measured after 24 h. Data are expressed as mean ± SD (n = 3). D: Pre-incubation with synthetic peptide blocks YSA-mediated targeting of human pancreatic cancer cell line MiaPaca-2. Cells were preincubated with 250 (grey bars) or 500 (black bars) μmol/L synthetic YSA peptide and transduced with 500 gc/cell of Ad-Luc/ΔF(FG)ΔP or Ad-Luc/ΔF(FG)ΔP-YSA. Luciferase was measured after 24 h. Data are expressed as mean ± SD (n = 3).

that, in this cell line, the EphA2R is present in nuclear granules. Since, in Capan-1 cells, the presence of the EphA2R on the membrane indicates that it is accessible for YSA binding, we chose to use this cell line for our subsequent studies.

Generation of ablated adenoviral vectors

We decided to use the FG loop mutation to ablate CAR binding because it has been well characterized[17,25]. By subsequent deletion of the RGD peptide from the penton base we obtained a doubly-ablated vector Ad-/ΔF(FG)ΔP which lacked both CAR and integrin binding. We inserted the YSA peptide in the HI loop of this doubly-ablated vector and generated Ad-/ΔF(FG)ΔP-YSA.

The lack of binding to native cellular receptors severely impairs the cellular entry of this doubly-ablated vector. Therefore, it can not be propagated on normal HEK 293 T cells. To overcome this, we used 293T cells that express the adenovirus type 5 fiber protein[20]. Incorporation of the normal fiber expressed in trans into the ablated vector allowed efficient cell entry and vector production. The last round of virus propagation was on normal 293T cells to generate the doubly-ablated vector. Because of impaired cell entry, the infectious particle (ip) titers of ablated vector stocks could not be preformed by standard procedures. To determine the functional titer of these vectors we therefore used a bi-specific antibody[23]. This antibody binds to the adenovirus knob and the epidermal growth factor receptor (EGFR). Upon binding to this antibody, the vector will enter the cells *via* the EGFR only. Therefore, this antibody allows direct comparison between the ip/gc ratio of the ablated and of the retargeted vectors.

To confirm the absence of CAR- and integrin-mediated entry, we compared the transduction efficiency of wild-type adenovirus type 5 with that of Ad-/ΔFΔP and Ad-/ΔFΔP-YSA on A549 cells that do express CAR but not EphA2R. The 3 to 4 log lower transduction by both ablated vectors confirmed efficient abolition of binding to the native receptors (Figure 2A). As expected, the transduction efficiency of both vectors on normal human fibroblasts that do not express the EphA2R was comparable but very low: 1.42×10^5 RLU for Ad-/ΔF(FG)ΔP and 1.77×10^5 RLU for Ad-/ΔF(FG)ΔP-YSA ($P = 0.06$) (Figure 2B). To confirm viability of the ablated vectors, we incubated both vectors with the bi-specific antibody, which resulted in a 2 log increase of

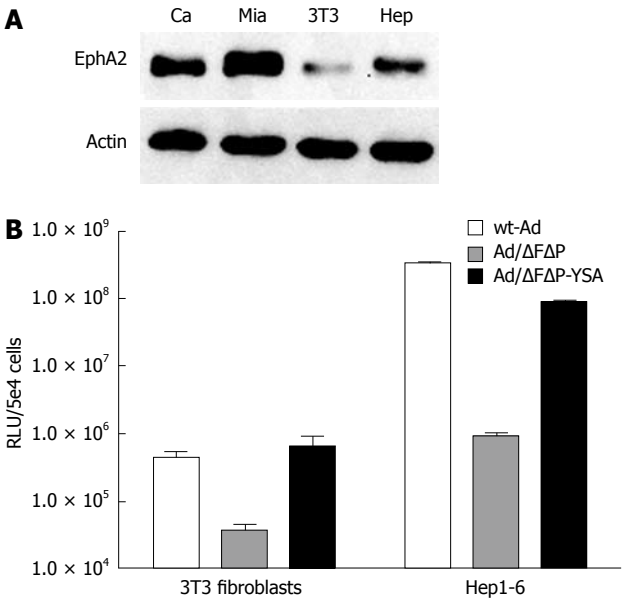

Figure 3 YSA targets adenovirus to cells expressing murine EphA2R. A: Western blotting demonstrates that mouse 3T3 and Hep1-6 cells, like human pancreatic cancer cell lines Capan-1 and MIA PaCa-2, express the EphA2R. EphA2R was detected using a monoclonal antibody and the housekeeping protein actin was used as a loading control. B: Efficient transduction of mouse 3T3 and Hep1-6 cells by YSA-retargeted adenoviral vector. Cells were transduced with 1000 gc/cell of wt-Ad-Luc (white bars), Ad-Luc/ΔF(FG)ΔP (gray bars) or Ad-Luc/ΔF(FG)ΔP-YSA (black bars). Luciferase was measured after 24 h. Data are expressed as mean ± SD (n = 3).

transduction efficiency for both vectors, Ad-/ΔF(FG)ΔP: 1.5×10^7 RLU and Ad-/ΔF(FG)ΔP-YSA: 1.9×10^7 RLU. The comparable transduction by both in the presence of the antibody indicated that both had a comparable ip/gc ratio ($P = 0.094$) and were viable.

To determine if insertion of the YSA peptide was functional, we tested transduction of both vectors on EphA2R-expressing Capan-1 and MiaPaca-2 cells. Although both cell lines do express integrins[7], ablation of native binding sites reduced their transduction by 3 to 1.5 logs. Insertion of the YSA peptide increased gene transfer to Capan-1 cells by 7.6-fold ($P = 0.0014$) and to MIA PaCa-2 cells by 4.5-fold ($P < 0.0001$). Insertion of the YSA peptide did not increase transduction in Hs766-T cells which only showed nuclear EphA2R staining (not shown). To confirm that entry of the retargeted vector was mediated by the YSA peptide, we performed competition experiments. As shown in Figure 2D, the 4-fold increased transduction efficiency of Ad-/ΔF(FG)ΔP-YSA compared to Ad-/ΔF(FG)ΔP was lost upon pre-incubation with synthetic YSA peptide. This indicated that the increased efficiency of the retargeted vectors was indeed mediated by the inserted YSA peptide. Thus, HI loop insertion of the YSA peptide in an adenovirus that lacks binding to CAR and integrins, resulted in cell entry *via* the EphA2R. Therefore, this vector appeared suitable for *in vivo* targeting of pancreatic cancer.

Targeting of the EphA2R by the YSA peptide has only been demonstrated in humans. Binding of the YSA peptide to the murine EphA2R has not been studied. Since binding to EphA2R expressed on activated (murine) endothelial cells will affect targeting of pancreatic cancer in a mouse model, we therefore decided to investigate the transduction efficiency of the YSA-retargeted adenoviral vector to mouse cells expressing the EphA2R. As shown in Figure 3A, mouse 3T3 fibroblasts and hepatoma cells (hepa1-6) both expressed the murine EphA2R, albeit at a lower level than in the human pancreatic cancer cell lines Capan-1 and MIA PaCa-2. Ablation of native cell binding sites reduced adenoviral transduction of these 2 mouse cell lines by 1 to 2 logs (Figure 3B). Insertion of the YSA peptide completely rescued cell entry of the ablated vector. Compared to the ablated vector, gene transfer of the YSA-retargeted vector was enhanced 17-fold in 3T3 cells and 95-fold in Hepa 1-6 cells. This efficient transduction of cells expressing the mouse EphA2R rendered the mouse a suitable model to study the efficiency of YSA-mediated targeting *in vivo*.

In vivo targeting in pancreatic cancer

To study *in vivo* targeting we used a *nu/nu* mouse model with subcutaneously growing human pancreatic tumor nodules. The virus was injected into mice which had nodules with a diameter of 0.4-0.7 cm, within 3 wk after injection of Capan-1 cells. We administered 1.0×10^{11} gc of ablated [Ad-/ΔF(FG)ΔP] or of the redirected vector Ad-/ΔF(FG)ΔP/-YSA, *via* the tail vein or i.p. Upon i.v. injection the clearance of both vectors was comparable. At 10 min after injection, $9.4 \times 10^8 \pm 6.3 \times 10^8$ gc/mL of Ad/ΔF(FG)ΔP and $1.1 \times 10^9 \pm 5.6 \times 10^8$ gc/mL of Ad/ΔF(FG)ΔP-YSA were present in blood. Since the total blood volume in a mouse is approximately 2.5 mL, the initial concentration of the virus was 4×10^{10} gc/mL. Thus 95% of the injected virus was cleared within 10 min. Based on the literature, we expected a slower clearance after i.p. injection, and determined the amount of virus in blood after 90 min. No significant difference was seen after 90 min between Ad-/ΔF(FG)ΔP-YSA ($1.4 \times 10^8 \pm 8.3 \times 10^7$ gc/mL) and Ad-/ΔF(FG)ΔP ($3.2 \times 10^8 \pm 4.3 \times 10^8$ gc/mL) ($P = 0.3$). Thus clearance for both vectors after *i.p.* injection was also comparable. Clearance of *i.p.*-injected virus was also efficient since less than 0.5% of the injected dose was present in blood after 90 min.

All mice were sacrificed 72 h after injection. Tissues were harvested and snap frozen in liquid nitrogen to determine adenoviral transduction by determining luciferase expression. After *i.v.* injection, expression of luciferase in the liver of Ad/ΔF(FG)ΔP-YSA treated mice was 6.7-fold lower ($P = 0.015$) than that in mice injected with Ad/ΔF(FG)ΔP (Figure 4B). Thus, the YSA peptide impaired liver transduction. No significant differences in expression were seen between both vectors in all other tissues. Surprisingly, the amount of luciferase expression in mice injected with Ad-/ΔF(FG)ΔP-YSA was only 15% of that in mice injected with Ad-/ΔF(FG)ΔP. This indicated that a large amount of the redirected vector was lost upon i.v. injection. The difference in luciferase expression between both vectors

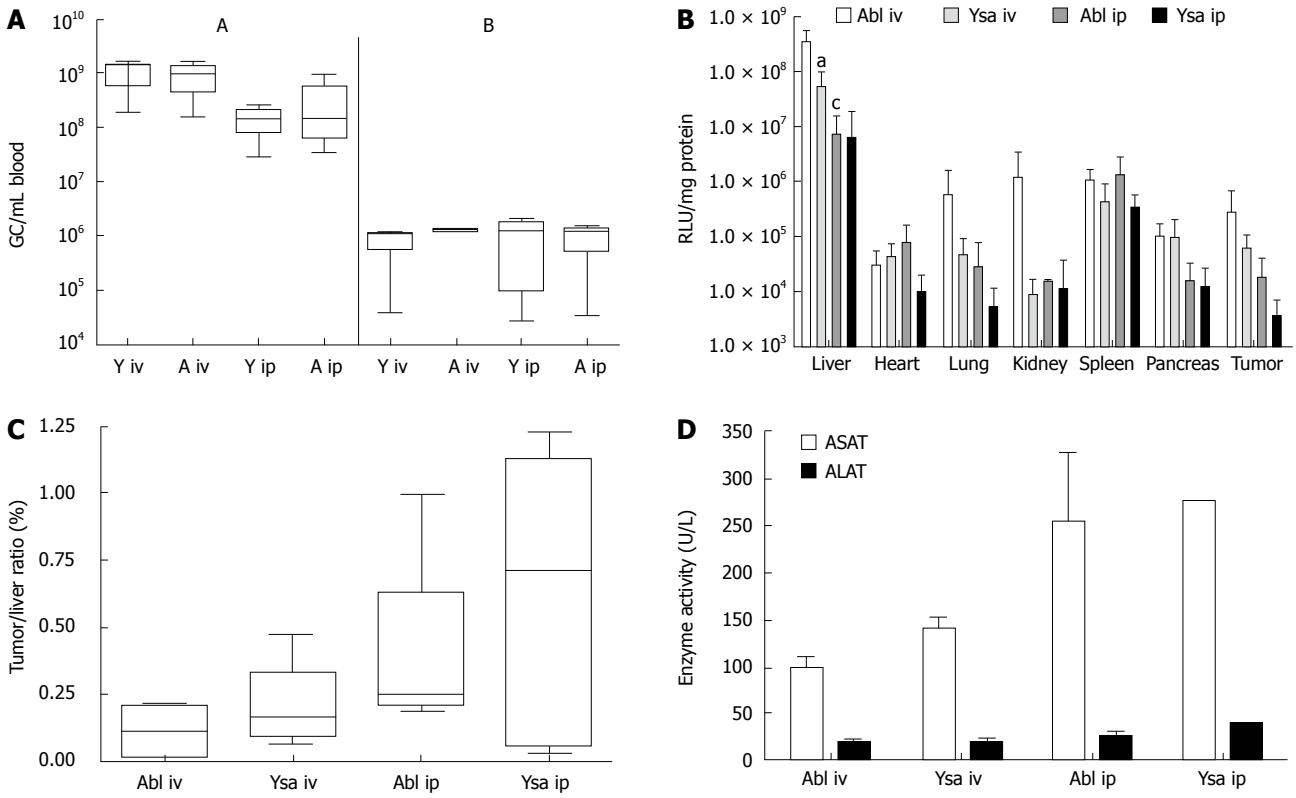

Figure 4 Lack of YSA specific targeting in nu/nu mice. A: 1 × 10^{11} gc of Ad/ΔF(FG)ΔP (ablated, A) or 1 × 10^{11} gc of Ad/ΔF(FG)ΔP-YSA (YSA; Y) are rapidly cleared from blood after intravenous (*i.v.*) and intraperitoneal (*i.p.*) administration into mice. At 10 min after *i.v.* or 90 min after *i.p.* injection and after 3 d (both), adenoviral genomic copies were determined in whole blood with real time PCR. B: Bio-distribution Ad/ΔF(FG)ΔP and Ad/ΔF(FG)ΔP-YSA injected *i.v.* or *i.p.* into *nu/nu* mice with a subcutaneous human pancreatic tumor. Animals were sacrificed 3 d after injection of 1 × 10^{11} gc of adenoviral vector. All organs were harvested and analyzed for luciferase expression/mg of protein. ($^{a}P < 0.05$ compared with Ad/ΔF(FG)ΔP after *i.v.* injection; $^{c}P < 0.05$ compared with Ad/ΔF(FG)ΔP after *i.v.* injection). C: The tumor/liver ratio of luciferase expression in each mouse demonstrates the lack of significant targeting of retargeted adenovirus to pancreatic cancer *in vivo*. D: Similar ASAT and ALAT levels in serum at 3 d after injection indicates comparable liver toxicity of ablated and retargeted adenoviral vectors in *nu/nu* mice. Data represent the mean ± SD of 4-7 mice.

was not seen after i.p. injection. This is due to the almost 50-fold lower luciferase expression in the liver after i.p. injection of Ad-/ΔF(FG)ΔP ($P = 0.0163$). For Ad/ΔF(FG)ΔP-YSA, the luciferase levels in the liver after *i.p.* injection were only reduced 9-fold and did not reach significance. The lower luciferase expression levels indicated that both vectors were cleared more efficiently without transduction after *i.p.* injection. To correct for the lower overall expression we chose to use the tumor liver ratio in each animal as an indication of retargeting efficiency. As shown in Figure 4C, no significant targeting of the tumor was seen with the YSA-redirected virus. Furthermore, the route of administration also did not affect tumor targeting.

Adenoviral vectors cause inflammation of the liver. Therefore we determined ALAT and ASAT levels 3 d after injection. Although we did see a 4 to 5-fold increase, no differences were seen between the 2 viral vectors (Figure 4D).

DISCUSSION

Conditional replicating adenovirus vectors are being developed to treating solid cancers[4,5]. However, these vectors have only been effective after direct injection into the tumors. As cancer is a systemic disease in virtually all fatal cases, this lack of systemic efficacy presents a major limitation to successful adenovirus-mediated gene therapy. Specific targeting therefore is a prerequisite for efficient eradication of solid cancer cells.

The aim of this study was to target the adenovirus to pancreatic cancers. Effective targeting *in vitro* to human cancer cells using HI loop insertion of peptide ligands has been reported by other groups. A well known example is the insertion of an integrin-binding RGD peptide that overcomes the poor transduction of human cancer caused by low expression of CAR[7,26,27]. Although integrins are highly expressed on cancer cells, the specificity of RGD targeting *in vivo* is questionable because of integrin expression in other tissues. Therefore more specific targeting peptides are needed. We compared binding of several ligands to receptors highly expressed on pancreatic cancers for their ability to target the adenovirus to pancreatic cancer cells (van Geer *et al*[12] in preparation). Of these ligands the YSA peptide appeared the most promising since it provided selective targeting *in vitro* to the EphA2R. Since YSA targets tissues expressing the EphA2R, we decided to test the specificity this retargeted vector *in vivo*.

Insertion of a peptide into the HI loop does not shield the native cell binding sites present in the adenoviral capsid. Binding of the retargeted vector

to these receptors present on liver cells for instance, will limit specific targeting[17]. Since ablation of these native binding reduces liver transduction and improves specific targeting *in vivo*[14], we combined YSA targeting with ablation of the native binding sites. We showed that ablation of CAR and integrin binding reduced adenoviral transduction by at least 2 logs *in vitro*. Insertion of the YSA peptide partly restored the transduction efficiency of the virus, but only on cells that expressed the EphA2R. Addition of synthetic YSA peptide blocked the increase in transduction efficiency. Together these data demonstrated that insertion of YSA enables EphA2R-mediated entry of ablated adenoviral vectors. Other groups have also reported that the loss of infectivity of ablated vectors can be restored by insertion of a targeting ligand in the HI loop[17,18,28]. However, not all retargeted vectors do provide efficient transduction. Insertion of RGD in a vector in which the CAR binding site was ablated in the KO1 mutation did not result in integrin-mediated uptake[29]. Apparently, in addition to preventing CAR binding, this mutation affected other essential steps such as internalization or trafficking of the adenovirus. In conclusion, our data and that of others show that HI loop insertion of a targeting peptide results in specific targeting of CAR/integrin-ablated adenoviral vectors *in vitro*.

Expression of the EphA2R is also enhanced in several normal tissues including tumor endothelium. Therefore, we investigated whether the YSA peptide also mediated adenoviral transduction *via* the mouse EphA2R. Since insertion of the YSA peptide increased the transduction of 2 mouse cell lines expressing the EphA2R by 1 to 2 logs, the YSA-retargeted vector was capable of targeting the mouse endothelium. The increase in transduction of mouse cells was stronger than in human pancreatic cancer cell lines while expression of the EphA2R in mouse cells was lower. This discrepancy seems to result from better accessibility of the EphA2R in mouse cells since, in human pancreatic cancer cells, most of the EphA2R was present in the cytosol (Figure 1A). Another possible explanation for this discrepancy is the expression of an inactive EphA2R in cancer cells. Since EphA2R activation impairs survival, cancer cells with an inactive receptor will have a growth advantage[11]. A third explanation could be a higher affinity of the Ad-/ΔF(FG)ΔP-YSA for the mouse EphA2R. Nevertheless, the efficient transduction of mouse cells expressing EphA2R renders the mouse a good model to study YSA-mediated targeting of pancreatic cancer *in vivo*.

After intravenous injection, luciferase expression in the liver of the YSA-retargeted vector Ad-/ΔF(FG)ΔP-YSA was lower than that by the ablated virus (Figure 4B). A decreased transduction of the liver was also reported in other studies in which the FG loop has been mutated. Thus, it seems that FG loop mutations lead to de-targeting of the hepatocytes[17]. In contrast, mutations in the AB loop did not decrease liver transduction[18,30]. These studies indicate that liver de-targeting occurred irrespective of the nature of the inserted peptide sequence. In our study, the decreased liver transduction due to YSA-retargeting was not accompanied by an increased transduction of any other tissue tested. Therefore, the increased loss of the redirected virus seems to result from degradation by tissue macrophages, which degrade more than 90% of injected adenoviruses. Since these cells do not express the EphA2R this would appear to be a non-specific effect.

In contrast to the receptor-mediated transduction of hepatocytes, uptake of adenovirus by macrophages depends on the binding of adenovirus fiber to blood factors. This induces uptake of adenovirus, for instance *via* the scavenger receptor[31,32]. Increased binding to blood factors of the mutated FG loop may cause the 10-fold greater loss of re-targeted adenovirus upon i.v. injection by increasing its degradation by macrophages. This may cause a lack of tumor targeting by Ad-/ΔF(FG)ΔP-YSA *in vivo*.

Akiyama *et al*[25] reported that after i.p. injection, a comparable ablated adenoviral vector efficiently entered the blood stream. Furthermore, they showed prolonged blood circulation and absence of hepatocyte transduction. Based on this, i.p. injection seemed a promising approach for systemic targeting. In our study, we could not repeat this observation. At 90 min after injection, less than 1% of the injected dose of vector was still present in the circulation while in contrast they still detected 20%. Increased uptake by macrophages may explain this discrepancy[33]. We used *nu/nu* mice to study retargeting of adenovirus while Akiyama *et al* reported a prolonged circulation time in normal mice. Several old studies have reported increased phagocytosis in *nu/nu* mice compared to normal mice to compensate for their immune defects[34,35]. A high dose of adenovirus can saturate the uptake by macrophages residing in the peritoneum and liver, and result in appearance of the virus in the circulation[36]. Apparently, the dose used in this study was too low to saturate the increased macrophage clearance capacity in *nu/nu* mice. The increased liver enzymes in serum after i.p. injection indicated increased macrophage uptake (Figure 4D). This increase was not observed for this ablated vector in normal mice. The increased uptake by macrophages in *nu/nu* mice rendered this model less suitable for adenoviral targeting studies[30]. Inhibition of phagocytosis therefore seems to be required to use this model for studying retargeting of ablated adenoviral vectors, e.g., by pre-injection of a small dose of virus[37].

For both administration routes, the luciferase expression in subcutaneously growing Capan-1 tumors was not significantly different between re-targeted and ablated vectors. The transduction of pancreatic cancer by our ablated vector suggests that adenoviral uptake can be mediated by other receptors. The role of additional receptors in Capan-1 cells is in accordance with Havenga *et al*[38]. They observed that gene transfer in these cells did not correlate with expression levels of CAR and/or integrins. In contrast, transduction by non-ablated adenoviral vectors was strongly impaired by loss

of CAR expression[7,39]. Apparently, removal of native cell binding sites is compensated by other low affinity binding sites, such as heparan sulphate proteoglycans[40]. The expression of the 2 most prominent proteoglycans, glypican-1 and syndecan-1, is indeed enhanced in pancreatic cancers. This may explain the efficient transduction of the tumor by the ablated vector[41,42]. Pancreatic cancer therefore seems susceptible to blood factor-mediated transduction by adenovirus as has been reported for herpes virus also[43]. Therefore, ablation of the sites in the fiber knob that bind to blood factors may be required to re-direct the adenovirus to cancer cells *in vivo*. Furthermore, studies to determine binding to (human) blood components of modified vectors are essential for predicting their *in vivo* efficacy. Binding to blood factors may explain why ablated vectors with a peptide insertion fail to target tumors following intravenous injection[6,44], while they do perform properly upon local injection[25,28,45].

In conclusion, we have generated a doubly-ablated virus that targets pancreatic cancer cells *via* the EphA2R. However, *in vivo* targeting remains inefficient as yet. Most likely, further modification of the Ad capsid is necessary to prevent binding to blood factors which lowers gene transfer to the liver[30,46].

COMMENTS

Background

The incidence of pancreatic adenocarcinoma is increasing in the Western world for unknown reasons. Due to its late diagnosis the prognosis for pancreatic cancer is very poor. Novel treatments such as adenovirus mediated gene therapy are needed to improve this.

Research frontiers

Adenoviral vector have been widely applied to treat solid tumors. Although the results in animal models were promising the results in subsequent clinical trials were disappointing due to limited transduction of the tumors. Poor transduction of cancer cells was mainly caused by their low expression of coxsackie and adenovirus receptor (CAR), the receptor that mediates adenoviral entry in to the cell.

Innovations and breakthroughs

Retargeting of adenoviral vectors can be used to circumvent CAR mediated entry and can improve the transduction of human cancer cells. In this study, the authors targeted adenoviral vectors to the EphA2 receptor that is highly expressed on pancreatic cancer cells *in vitro* and *in vivo*. To further improve specific targeting to cancer cells they removed the regions in the adenoviral capsid that mediate transduction of the liver. *In vitro*, this strategy indeed proved very specific. The results in a nude mouse model were however disappointing most likely due increased uptake of the retargeted vector by macrophages, a route that is enhanced in these mice due to a compensatory mechanism for loss of other immune responses.

Applications

The authors show that adenovirus can be targeted to the EphA2 receptor *in vitro*. However to allow application *in vivo* further ablation of endogenous adenobinding sites seem needed.

Terminology

EphA2 or ephrine A2 receptor is a receptor involved in embryogenesis and is upregulated in several solid tumors. Adenovirus has a icosahedral symmetry with 12 fibers spiking out at all corners. The adenofibers are trimeric proteins. At the end they form a knob with a peptide stretch exposed to the surface, the HI-loop.

Peer review

The goal of this study was to generate an adenoviral vector specifically targeting the EphA2 receptor, which is highly expressed on pancreatic cancer cells *in vivo* by first using an *in vitro* model. The methodology incorporated into this particular study was sound and that their findings were novel.

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Gallbladder function and dynamics of bile flow in asymptomatic gallstone disease

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Abstract

AIM: To investigate the effects of gallbladder stones on motor functions of the gallbladder and the dynamics of bile flow in asymptomatic gallstone disease.

METHODS: Quantitative hepatobiliary scintigraphy was performed to detect the parameters of gallbladder motor function [gallbladder ejection fraction (GBEF), gallbladder visualization time (GBVT), gallbladder time to peak activity (GBT_{max}), gallbladder half emptying time ($GBT_{1/2}$), and transit time of bile to duodenum (TTBD)] in 24 patients with asymptomatic cholelithiasis who were diagnosed incidentally during routine abdominal ultrasonographic examination and 20 healthy subjects with normal gallbladder.

RESULTS: Even though there was no significant difference in the clinical and laboratory parameters between the patient and control groups, all parameters of gallbladder function except TTBD were found to differ significantly between the two groups. GBEF in the patient group was decreased (P = 0.000) and GBVT, GBT_{max}, $GBT_{1/2}$ in the patient group were longer (P = 0.000, P = 0.015, P = 0.001, respectively).

CONCLUSION: Our results showed that even if there were not any clinical and laboratory findings, gallbladder filling and emptying could be impaired in patients with gallstone disease.

Key words: Asymptomatic gallstone disease; Hepatobiliary scintigraphy; Gallbladder function

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INTRODUCTION

Asymptomatic cholelithiasis is being diagnosed increasingly, mainly as a result of the widespread use of abdominal ultrasonography for the evaluation of patients for unrelated or vague abdominal complaints and in cases of routine checkup. Most studies have indicated that the progression of asymptomatic to symptomatic disease is relatively low[1-4]. Despite some controversy most authors agree that the vast majority of subjects should be managed by observation alone. The major concern when discussing the natural history of asymptomatic cholelithiasis is the possible development of a severe, potentially life-threatening complication, such as acute supurative cholangitis, severe pancreatitis, cholecystoenteric fistula, gallstone ileus or rarely gallbladder cancer. Unfortunately, it is impossible, using local (such as number, size, nature, alteration in wall thickness or gallbladder contractility) or general factors (such as age, gender, or associated comorbidity) to predict who among asymptomatic patients, will develop symptoms or complications and when[5].

Hepatobiliary scintigraphy is used to show both morphological and physiological changes in the gallbladder. Since physiological changes usually precede

morphological alterations by several weeks or months, there is great potential for early diagnosis by scintigraphy, before irreversible functional changes take place[6]. The main advantage of hepatobiliary scintigraphy is that the technique is noninvasive, quantitative, and reproducible and has a low interobserver error rate[7-11].

The current study aimed to investigate by quantitative hepatobiliary scintigraphy the effects of gallbladder stones on motor function of the gallbladder and the dynamics of bile flow in a group of patients with asymptomatic gallstone disease.

MATERIALS AND METHODS

The study design was approved by the local University ethical committee and was performed according to the Helsinki Declaration. Informed written consent was obtained from all participating subjects before their involvement in the study.

Subjects

The study was conducted from April 2006 to February 2008, and included 25 patients with asymptomatic cholelithiasis who had been diagnosed incidentally during routine abdominal ultrasonography. There were no gallstone-related symptoms, such as history of biliary pain (pain in the epigastrium or right upper abdominal quadrant that may radiate to the patient's back or to the right scapula) or gallstone related complications such as acute cholecystitis, cholangitis, or pancreatitis. The only one patient with nonvisualized gallbladder during hepatobiliary scintigraphy was excluded. Twenty-four patients, (10 male and 14 female; aged 54.66 ± 12.59 years) with asymptomatic gallbladder stones, and 20 control cases (12 male, 8 female; aged 50.30 ± 4.15 years) with normal gallbladder were enrolled in the study. None of the subjects had diabetes mellitus, or a history of disease or operation that affected gallbladder motility. None of the patients had received recent medication such as cholic acid, morphine, atropine, calcium channel blockers, octreotide, progesterone, indomethacin, theophyline, benzodiazepines, and histamine-2 receptor antagonists to influence gallbladder motor function. All patients in the study and control group had normal gallbladder wall thickness (no more than 2 mm), common bile duct upon ultrasound examination and liver function as shown by routine biochemical screening measures [aspartate amino transferase (AST), alanine aminotransferase (ALT), γ-glutamyltransferase (GGT), alkaline phosphatase (ALP) and total bilirubin levels].

Hepatobiliary scintigraphy

After the patients had fasted overnight, hepatobiliary scintigraphy was performed using 185 MBq (5 mCi) of 99m-Tc-mebrofenin (BRIDATEC, GIPHARMA S.r.I., Italy) intravenously. Two-phase dynamic images were taken from the right hypochondrium with the patient in the supine position, using a dual-head gamma camera (Siemens E-CAM, Illinois, USA) which included a low-energy high resolution collimator. Phase 1: 2 s × 60 frames (perfusion phase); phase 2: 60 s × 118 frames (hepatobiliary phase). In the mid-term of the second phase, a standard fatty meal (100 g milk chocolate) instead of cholecystokinin was given to the patients in order to stimulate gallbladder contraction. All of the dynamic images were evaluated with the raw data and cine projections from the computer.

We obtained the following parameters. (1) Gallbladder ejection fraction (GBEF) was calculated by determining count variation in the gallbladder during the filling and emptying period, using a computer program for GBEF. An E-CAM Siemens computer program calculated GBEF according to the time variation curves of these two phase (Figure 1). (2) Gallbladder visualization time (GBVT). (3) Gallbladder time to peak activity (GBT_{max}). (4) Gallbladder half emptying time ($GBT_{1/2}$). (5) Transit time of bile to duodenum (TTBD) were evaluated.

Table 1 Clinical and laboratory features of patient and control groups (mean ± SD)

	Patients (n = 24)	Controls (n = 20)	P
Number (M/F)	24 (10/14)	20 (12/8)	0.345
Age (yr)	54.66 ± 12.59	50.30 ± 4.15	0.267
AST (U/L)	30.62 ± 13.89	27.30 ± 9.99	0.547
ALT (U/L)	34.66 ± 27.86	21.60 ± 5.71	0.283
GGT (U/L)	48.20 ± 26.63	43.65 ± 10.80	0.915
ALP (U/L)	86.20 ± 23.68	86.10 ± 24.03	0.972
Total bilirubin (mg/dL)	0.74 ± 0.28	0.69 ± 0.27	0.579

Statistical analyses

The statistical analyses were done using SPSS 13 for Windows (Chicago, IL, USA). The data of the groups were given as mean ± SD and the Mann-Whitney U test was used as a non-parametric test to compare the means between the groups. $P < 0.05$ was considered as significant.

RESULTS

Table 1 shows the clinical and laboratory features of the patient and control groups (mean ± SD). There was no statistically difference in the clinical and laboratory parameters between the patient and control group ($P > 0.05$).

GBEF, GBVT, GBT_{max}, $GBT_{1/2}$, and TTBD of the patient and control groups are shown in Figure 2A-E. Mean GBEF in the patient group decreased when compared with that in the control group (49.79 ± 25.42 min *vs* 78.20 ± 11.23 min; $P = 0.000$). Mean GBVT (21.83 ± 8.51 min *vs* 12.20 ± 2.28 min; $P = 0.000$), GBT_{max} (59.41 ± 15.09 min *vs* 49.30 ± 6.74 min $P = 0.015$), $GBT_{1/2}$ (99.37 ± 22.95 min *vs* 74.40 ± 11.12 min $P = 0.001$) were longer in the patient group than in the control group. There was no significant difference in mean TTBD (22.58 ± 14.08 min *vs* 27.00 ± 15.36 min) between the two groups.

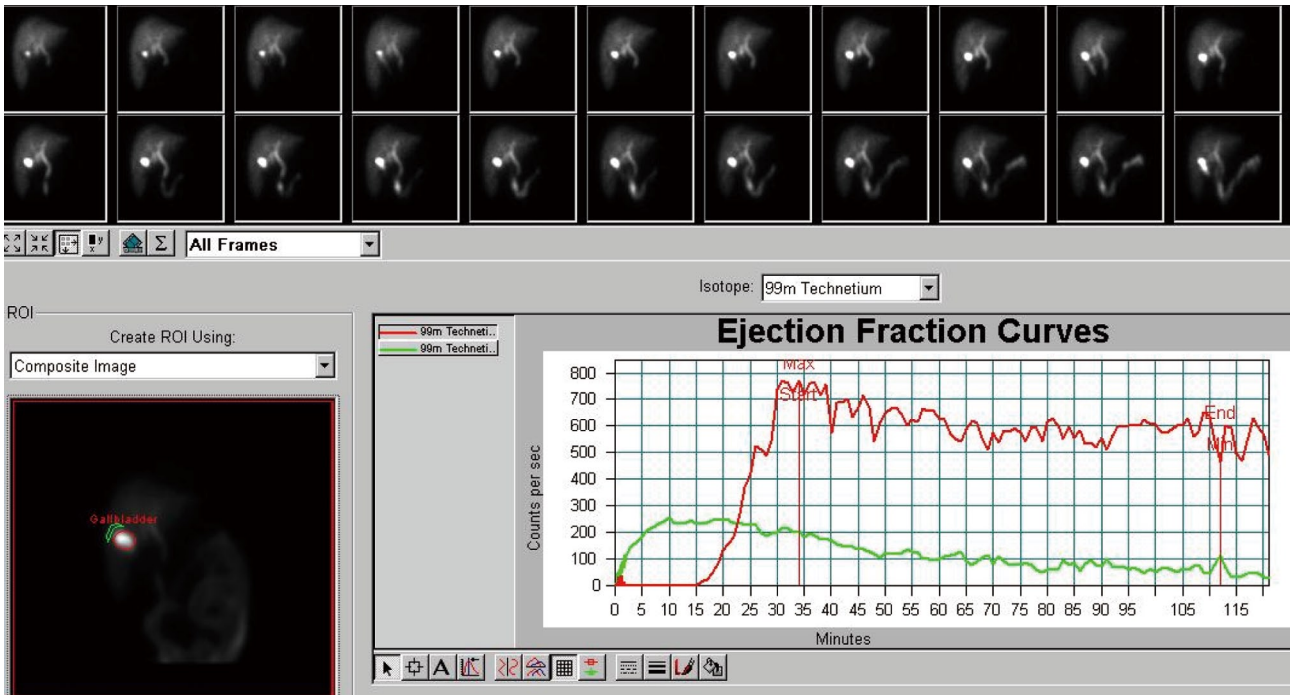

Figure 1 Image from Tc-99m mebrofenin cholescintigraphy and GBEF curve in a patient with asymptomatic cholelithiasis. Gallbladder emptying was slower and was not completed during the study.

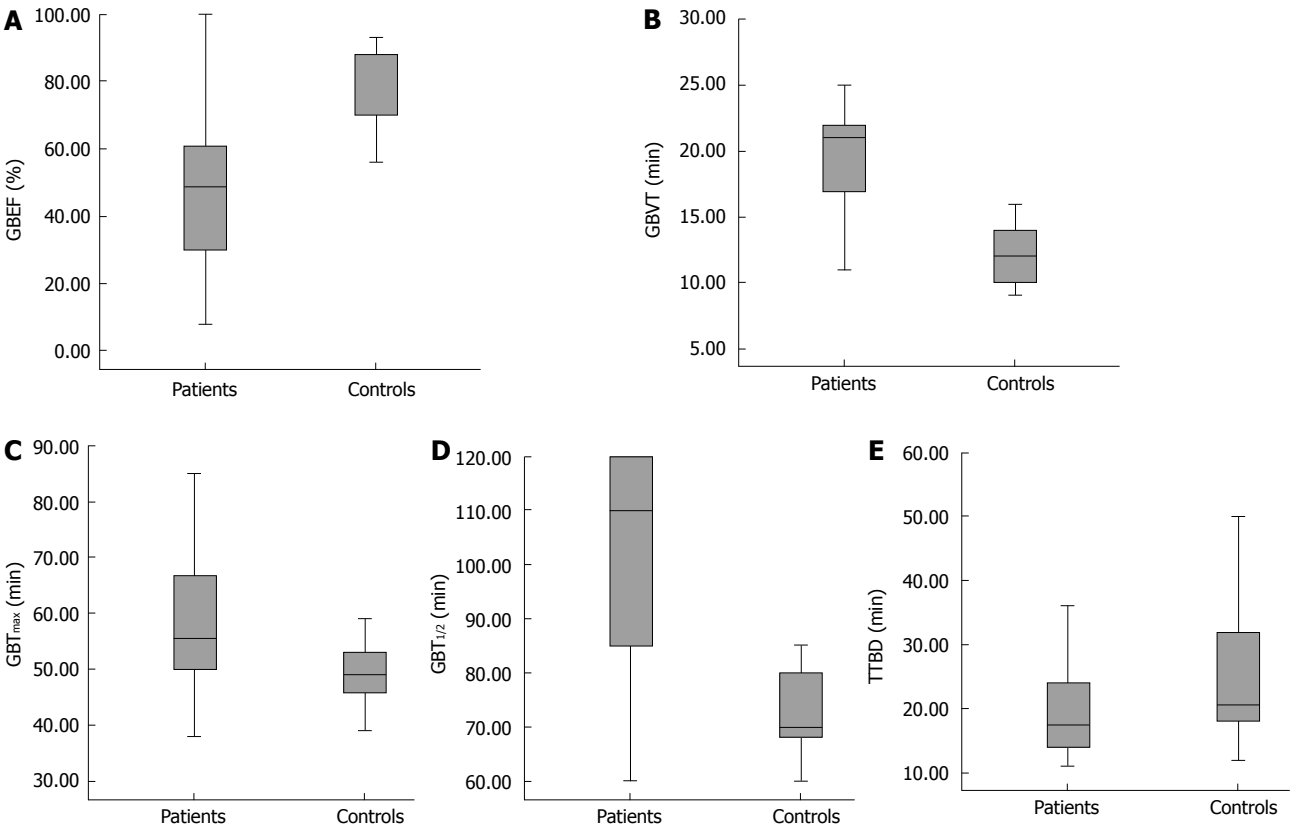

Figure 2 Parameters of gallbladder function. A: Mean GBEF of the patient group was significantly decreased when compared with the control group (P = 0.000); B: Mean GBVT was significantly longer in asymptomatic gallbladder patients than in the control group (P = 0.000); C: GBT_{max} was significantly longer in the patient group (P = 0.015); D: $GBT_{1/2}$ was significantly prolonged in the patient group (P = 0.001); E: There was no significant difference between TTBD in the two groups.

DISCUSSION

In the past, the majority of the literature has focused on the pathogenesis of gallstone formation rather than on the association of gallbladder motility and clinical symptoms[12]. Gallbladder stones may be asymptomatic in a considerable number of patients, and the pathogenesis of symptoms is not understood clearly.

Theoretically, gallbladder function may be an important predictor of outcome from either cholecystectomy or watchful waiting, because the symptoms traditionally are believed to arise from gallbladder contraction[13].

Several studies using different techniques and protocols have assessed gallbladder function in gallstone disease[8,14-16]. In these studies, patients groups were generally taken from symptomatic but uncomplicated patients.

Gallbladder emptying is under the control of neural and hormonal stimulation. For normal bile flow, Oddi sphincter relaxation should synchronize with gallbladder contraction. CCK, as a mediator, is responsible for relaxation of the sphincter of Oddi and gallbladder contraction. After a fatty meal is eaten, the gallbladder empties with active contraction, which is regulated mainly by the release of endogenous CCK, which simultaneously induces Oddi sphincter relaxation, therefore allowing maximal bile outflow from the common bile duct into the duodenum at the time of maximal gallbladder contraction[17].

In this manner, it is important to understand how symptoms occur and what the reason is. It is also important to know that, if we perform cholecystectomy the pathological bile flow will resolve. It is still controversial whether impaired gallbladder emptying/contraction is the cause or the result of bile stones[13].

Quantitative hepatobiliary scintigraphy is a well-established method that can be used in the evaluation of hepatocellular function and patency of the biliary system by tracing the production and flow of bile from the liver through the biliary system into the small intestine[18]. Of the quantitative parameters of hepatobiliary scintigraphy, time variables of the gallbladder (GBVT, GBT_{max}, $GBT_{1/2}$, and TTBD) and GBEF are regarded as sensitive parameters for diagnosing gallbladder motor function abnormalities.

In previous scintigraphic studies, GBEF and gallbladder emptying time were found to be different in patients with symptomatic gallbladder stones[14,15,19]. Most of these studies, agreed that, although gallbladder emptying was impaired, filling was unaffected. In our study, emptying time was significantly longer in patients with asymptomatic gallstones and GBEF was significantly reduced.

We found that gallbladder filling time was also prolonged compared with the controls and Kao *et al*[20] have reported that gallbladder stones may impair gallbladder function, especially the filling fraction. Abnormal gallbladder filling and emptying of bile in the gallbladder can result from mechanical obstruction to bile flow, such as altered cystic duct resistance or abnormal sphincter of Oddi tone, decreased gallbladder contractile force, or increased bile viscosity. Patients with organic obstruction at the cystic duct could not be visualized during hepatobiliary scintigraphy and therefore a patient who had a non-visualized gallbladder was excluded from the present study. Increased resistance to bile flow might occur either at the cystic duct or sphincter of Oddi. In our study, there was no difference in TTBD between the control group and asymptomatic gallstone group, thus increased resistance to bile flow in the sphincter of Oddi was not the cause of prolongation in emptying time. On the other hand, viscosity tends to be higher in gallbladder bile of patients with gallstones[21] and may be another cause abnormal gallbladder emptying or filling, but normal TTBD was probably the indicator of normal bile viscosity in our study group. The most likely explanation for the abnormal gallbladder filling in our patients was increased resistance to bile flow at the cystic duct. Similar to our findings Pitt *et al*[22] have reported increased cystic duct resistance in rodents with gallstones, but we have not been able to find any human study about cystic duct resistance in patients with gallbladder stones. Jazrawi *et al*[23] have combined ultrasonography with scintigraphy and have shown that turnover of bile is impaired during the refilling phase in patients with gallstones. Moreover Cicala *et al*[24] have demonstrated that there is decreased turnover of bile that may contribute to cholesterol crystal precipitation and stone growth, as shown by ultrasonographic measurements of gallbladder volume variation. From another point of view, in the patient group, abnormal gallbladder smooth muscle contraction was probably the cause of both impaired emptying time and reduced GBEF.

It is also known that, in patients with impaired emptying, the contractile defect may have developed at a very early stage of gallstone formation[25]. Furthermore, the symptoms in gallstone patients are believed traditionally to arise from gallbladder spasm and normal gallbladder contractility is thought to be a prerequisite for the development of symptoms[12]. The gallbladder motility defect is restricted apparently to asymptomatic patients and appears to protect from symptomatic disease[26].

In conclusion, our results showed that even if there were not any clinical and laboratory findings, gallbladder filling and emptying can be impaired in gallstone patients.

COMMENTS

Background

Asymptomatic cholelithiasis is being increasingly, diagnosed today, mainly as a result of the widespread use of abdominal ultrasonography. Hepatobiliary scintigraphy is a noninvasive, quantitative, and reproducible technique that can be used to show morphological and physiological changes in the gallbladder. The authors investigated by hepatobiliary scintigraphy the effects of gallbladder stones on motor function of the gallbladder and the dynamics of bile flow in asymptomatic gallstone disease.

Research frontiers

Cholelithiasis is a very common disease, and it is still controversial whether impaired gallbladder emptying/contraction are the cause or result of bile stones. Gallbladder stones may be asymptomatic in a considerable number of patients with gallstones, and the pathogenesis of symptoms is not understood clearly. In previous scintigraphic studies, motor function parameters of the gallbladder have been found to be different in patients with symptomatic gallbladder stones, however, no definitive data have been published in asymptomatic cholelithiasis.

Innovations and breakthroughs

The authors showed for the first time that, even in the absence of any clinical and laboratory findings, gallbladder motor functions such as filling and emptying time and ejection fraction, were impaired in asymptomatic gallstone patients.

Applications

Their study was designed to analyze the scintigraphic parameters of gallbladder motor function (gallbladder ejection fraction, gallbladder visualization time, gallbladder time to peak activity, gallbladder half emptying time, and transit time of bile to duodenum) in patients with asymptomatic cholelithiasis who had been diagnosed incidentally during routine abdominal ultrasonography.

Terminology

99m-Tc-mebrofenin is a radiopharmaceutical agent for hepatobiliary scintigraphy, Gallbladder ejection fraction describes gallbladder emptying function.

Peer review

This is a very interesting study. This paper reports on the results of an investigation aimed at assessing the effects of gallbladder stones on gallbladder motility and at assessing the dynamics of bile flow in asymptomatic gallstone disease patients. The authors report that, even in the absence of any clinical and laboratory findings, gallbladder filling and emptying can be impaired in this subgroup of gallstone patients.

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BRIEF ARTICLES

Application of a biochemical and clinical model to predict individual survival in patients with end-stage liver disease

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Abstract

AIM: To investigate the capability of a biochemical and clinical model, BioCliM, in predicting the survival of cirrhotic patients.

METHODS: We prospectively evaluated the survival of 172 cirrhotic patients. The model was constructed using clinical (ascites, encephalopathy and variceal bleeding) and biochemical (serum creatinine and serum total bilirubin) variables that were selected from a Cox proportional hazards model. It was applied to estimate 12-, 52- and 104-wk survival. The model's calibration using the Hosmer-Lemeshow statistic was computed at 104 wk in a validation dataset. Finally, the model's validity was tested among an independent set of 85 patients who were stratified into 2 risk groups (low risk $\leqslant$ 8 and high risk > 8).

RESULTS: In the validation cohort, all measures of fit, discrimination and calibration were improved when the biochemical and clinical model was used. The proposed model had better predictive values (c-statistic: 0.90, 0.91, 0.91) than the Model for End-stage Liver Disease (MELD) and Child-Pugh (CP) scores for 12-, 52- and 104-wk mortality, respectively. In addition, the Hosmer-Lemeshow (H-L) statistic revealed that the biochemical and clinical model (H-L, 4.69) is better calibrated than MELD (H-L, 17.06) and CP (H-L, 14.23). There were no significant differences between the observed and expected survival curves in the stratified risk groups (low risk, $P = 0.61$; high risk, $P = 0.77$).

CONCLUSION: Our data suggest that the proposed model is able to accurately predict survival in cirrhotic patients.

Key words: Liver cirrhosis; Prognosis; Statistical models; Prognostic factors; Model for end-stage liver disease score; Child-Pugh score; Survival

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INTRODUCTION

The Model for End-stage Liver Disease (MELD) and Child-Pugh (CP) scores have been the most widely applied prognostic markers for organ allocation in liver transplantation, mainly due to their simplicity of use in daily clinical practice[1-5]. The MELD score has gained wide acceptance for predicting survival in patients undergoing liver transplantation. It has been suggested

that it provides more accurate prognosis than the Child-Pugh (CP) score in patients with decompensated cirrhosis and that it therefore improves the evaluation of priority for liver graft allocation[4,5]. It is not surprising, however, that the magnitude of superiority of the MELD score over the CP score is modest and is primarily limited to the population at the highest risk of renal failure[6]. Additionally, changes in some objective laboratory parameters of the MELD score may be directly related to the extensive use of diuretics, volume status, albumin infusion and the patient's nutritional status. Finally, clinical complications of portal hypertension such as ascites, encephalopathy, spontaneous bacterial peritonitis (SBP) and gastrointestinal bleeding are not considered in the MELD score, probably underestimating any direct association with the severity of liver disease[7]. However, the model has been shown to predict mortality independent of the occurrence of complications of portal hypertension[3,4]. The classification applied to the clinical complications of portal hypertension (ascites, encephalopathy, variceal bleeding and SBP) in the MELD score does not clearly reveal the different grades of severity of liver disease and its clinical response to medical treatment. Therefore, its utility as a prognostic model could be limited. In this regard, several recent studies have shown that clinical manifestations secondary to portal hypertension (encephalopathy, ascites) are good prognostic markers in cirrhotic patients[8,9]. According to the results of these studies, the use of clinical markers in prognostic models may be recommended.

The aim of this study was to evaluate the short-, medium- and long-term prognosis of a series of cirrhotic patients by means of the BioCliM score using biochemical (creatinine and bilirubin) and clinical (encephalopathy, bleeding esophageal varices and ascites) variables, to compare BioCliM with the MELD and CP scores, and to identify those variables with liver-related mortality. Our model was developed to improve accuracy in predicting survival and consequently improve the further evaluation of priority for liver graft allocation in cirrhotic patients.

MATERIALS AND METHODS

Study design, setting and participants

We prospectively evaluated 180 consecutive cirrhotic patients who were admitted at the National Institute of Gastroenterology of Havana during the period May 2003 to January 2006. Inclusion criteria were histological, laparoscopic or clinical diagnosis of cirrhosis and presence of compensated or decompensated disease (stages A, B or C according to the CP classification). Patients with hepatocellular carcinoma, severe infection, severe primary cardiopulmonary failure, alcohol use within one month before initial evaluation, and intrinsic kidney disease were excluded from the study. Among 180 patients who had complete medical profiles and an established diagnosis of hepatic cirrhosis, 172 patients fulfilled the above selection criteria.

The model validation was performed by applying it to an independent group of 85 patients who were evaluated at the "Calixto Garcia" Hospital of Havana from March 2005 to August 2007. The baseline characteristics of the patient population are summarized in Table 1.

Table 1 Baseline characteristics of the patient population

Variables	Derivation set *n* = 172	Validation set *n* = 85
Follow-up period	56 (4-104)	58 (8-104)
Age (yr)	56 (20-79)	59 (23-78)
Sex, *n* (%)		
Male	106 (62)	58 (68)
Female	66 (38)	27 (32)
Cause of cirrhosis, *n* (%)		
Alcohol	30 (17)	16 (19)
Alcohol plus viral infection	15 (9)	4 (5)
HBV	20 (12)	12 (14)
HCV	92 (53)	50 (59)
Viral co-infection (HBV/HCV)	1 (1)	1 (1)
Unknown	13 (7)	1 (1)
NAFL	1 (1)	1 (1)
Complications on admission, *n* (%)		
Ascites		
Absent or controlled	147 (85)	72 (85)
Uncontrolled	25 (15)	13 (15)
BEV		
Absent or present without relapses	167 (97)	79 (94)
Present with relapses	5 (3)	5 (6)
Encephalopathy		
Absent or controlled	160 (93)	79 (93)
Uncontrolled	12 (7)	6 (7)
SBP		
Absent or present without relapses	168 (98)	82 (96)
Present with relapses	4 (2)	3 (4)
Hepatorenal syndrome, *n* (%)	3 (2)	1 (1)
Prothrombin time (s)	19 (13-55)	17 (13-53)
Partial thromboplastin time (s)	38 (26-165)	39 (26-167)
INR for prothrombin time	1.7 (1-7.5)	1.5 (1-6.9)
Albumin (g/L)	37 (20-48)	36 (21-47)
Creatinine (mmol/L)[1]	100 (42-516)	98 (39-489)
Bilirubin (mmol/L)[2]	20 (8-130)	23 (12-137)
Cholesterol (mmol/L)	3.8 (1.9-10.2)	3.9 (2-9.6)
Child-Pugh score[3]	7 (5-14)	7 (5-14)
Child-Pugh A, *n* (%)	67 (39)	30 (35)
Child-Pugh B, *n* (%)	75 (44)	34 (40)
Child-Pugh C, *n* (%)	30 (17)	21 (25)
MELD score	17 (9-42)	18 (10-43)
BioCliM score	7.7 (6.1-13.6)	7.9 (6-13.8)

HBV: Hepatitis B virus; HCV: Hepatitis C virus; NAFL: Non-alcoholic fatty liver; BEV: Bleeding esophageal varices; SBP: Spontaneous bacterial peritonitis; INR: International normalized ratio; MELD: Model for End-stage Liver Disease; BioCliM: Biochemical and Clinical Model. All quantitative variables are expressed as median (ranges). [1]To convert mmol/L into mg/dL, multiply by 0.01131. [2]To convert mmol/L into mg/dL, multiply by 0.0585. [3]The Child-Pugh, MELD and BioCliM scores are measures of the severity of liver disease.

Variables of interest, measurement, follow-up and ethics

Detailed medical history, complete physical examination, and a battery of laboratory tests were performed in all patients on the day of admission. Biochemical evaluations were carried out by the same laboratory. Prothrombin time expressed as PT-ratio (patient-to-normal coagulation time) was converted to prothrombin time international normalized ratio (INR) using an internal laboratory standard and was assessed by a single

operator. The main clinical complications of portal hypertension were initially evaluated and classified by an experienced hepatologist depending on the clinical response to medical treatment. Bleeding esophageal varices (BEV) were diagnosed by clinical signs of hematemesis and endoscopic signs of active bleeding or adherent clots on EV[10]; they were classified as absent, present with relapses or rebleeding (2 or more bleeding episodes in the last 3 mo) or without relapses (one bleeding episode in the last 3 mo). Variceal bleeding relapse or rebleeding was defined as the occurrence of hematemesis/melena, aspiration of more than 100 mL of fresh blood in patients with a nasogastric tube and decrease of 3 g in Hb if no transfusion was given. Portosystemic encephalopathy was defined according to the West Haven criteria for grading from 0 (subclinical) to 4 (coma)[11]; it was classified as absent (no episode of encephalopathy in the last year), medically controlled (episodic hepatic encephalopathy developing over hours to days, but does not persist with adequate medical treatment) or uncontrolled (persistent hepatic encephalopathy that develops upon discontinuation of medication), irrespective of disease severity. Ascites was classified as absent (no clinical and ultrasound evidence of ascites and without therapeutic intervention), medically controlled (no clinical and ultrasound evidence of ascites in patients undergoing full therapeutic intervention) or uncontrolled (ascites that requires repeated paracentesis for control or a sodium-restricted diet and intensive diuretic therapy). Diagnostic paracentesis and ascitic fluid culture were performed in all admitted cirrhotic patients. Spontaneous bacterial peritonitis (SBP) was diagnosed when the ascites polymorphonuclear leukocyte (PMN) count was > 250/mm^3, with or without positive ascites bacterial culture[12]; it was coded as absent, present without relapses (one SBP episode in the last year) and present with relapses (2 or more episodes in the last year). Patients were followed up from their date of initial evaluation until death (related or unrelated to liver disease), liver transplantation, or study closure. Patients with death unrelated to liver disease were excluded from the analysis. Patients lost to follow-up were censored at the last date known to be alive and patients undergoing liver transplantation were censored at the transplant date.

The study was conducted in compliance with the Declaration of Helsinki and approved by the ethics committee and the institutional review board of the National Institute of Gastroenterology. All patients provided written informed consent for participation.

Analysis for survival and derivation of the novel risk score

The probable prognostic predictors, including age, sex, serum biochemistry and clinical complications of portal hypertension, were analyzed to determine prognostic ability. To lessen the influence of extreme laboratory values, quantitative variables were transformed to their natural logarithms.

Univariate and multivariate forward stepwise Cox proportional hazards models were used to determine variables associated with survival. Variables that were significant ($P < 0.05$) in univariate analysis were included in multivariate analysis. Stepwise probabilities for entry or removal were set at 0.05 and 0.10, respectively.

With each Cox model, a risk score for each patient was calculated as: $R = \beta_1 X_1 + \beta_2 X_2 + \ldots + \beta_k X_k$, where X_1, X_2,…, X_k are the values of prognostic factors and β_1, β_2, …, β_k are the corresponding regression coefficients. A higher risk score corresponds to poorer prognosis.

The forward stepwise selection procedures were used for variable selection, assessment for interactions, and model development. The likelihood ratio statistic tested the significance of the addition of each variable separately to a predictive model that included ascites only. Furthermore, the c-statistic was computed as a criterion for the selection of a group of variables to be used in the new predictive model. The final criterion for inclusion in the model was minimization of the Bayes Information Criterion (BIC)[13]. The BIC is a likelihood-based measure in which lower values indicate better fit and in which a penalty is paid for increasing the number of variables. Thus, the variables selected for inclusion should provide not only the best fit but also a parsimonious prediction model.

Predictive models for survival and discrimination

The CP and MELD scores were calculated on parameters obtained at referral. The MELD score was calculated according to the original formula proposed by the Mayo Clinic group as follows: [9.57 × $\log_e$ creatinine mg/dL + 3.78 × $\log_e$ bilirubin mg/dL + 11.20 × $\log_e$ INR + 6.43 (constant for liver disease etiology)]. To avoid negative scores, laboratory values less than 1 were rounded up to 1. The maximal value of creatinine was 4 mg/dL[3].

Once a new risk model was determined, it was prospectively tested in the validation dataset of 85 patients from "Calixto Garcia" Hospital. The discrimination ability of the different models was measured by means of the concordance statistic (c-statistic), a measure of discrimination also known as a natural extension of the receiving operator characteristic (ROC) curve area in survival analysis. *P* values for the comparison of the c-statistic were computed using the bootstrap method. A c-statistic between 0.8 and 0.9 indicates excellent accuracy, and a value over 0.7 should be considered clinically useful. The concordance c-statistic was assessed for 12-, 52-, and 104-wk survival. A time-to-event with the censored data version for survival analysis was performed to compute the c-statistic.

The concordance probability estimates (CPE) were computed[14], because the c-statistic seems to overestimate the true concordance probability, especially if the censoring proportion is high. Since the CPE is a consistent estimate, it is a better measure in the context of using predictions from Cox regression models.

Calibration and external validation of the new risk score

To assess model calibration (or how closely the predicted

probabilities reflect actual risk), the Hosmer-Lemeshow calibration statistic, as modified by D'Agostino *et al*[15], comparing observed and predicted risk was implemented.

In addition, the new risk model was validated in a cohort of 85 independent patients from "Calixto Garcia" Hospital who were stratified into 2 risk (R) groups: R $\leqslant$ 8 and R > 8. Within each risk group the survival was calculated using the Kaplan-Meier procedure and the observed-predicted survivals were compared using the log-rank test.

Analyses were performed with the use of SAS software, version 9.1 (SAS Institute).

RESULTS

A total of 180 patients were examined for eligibility, and 172 were included in the study. The reasons for non-participation were: 3 patients with HCC, 3 with repeated alcohol use, and 2 with severe infection disease. The period of recruitment lasted from May 2003 to February 2004. One hundred and forty one patients completed the follow-up period. Thirty one patients died during the study, 29 liver-related and 2 unrelated to liver disease (myocardial infarction). One hundred and seventy patients were included in the outcome analyses.

The patients' clinical and serological features are summarized in Table 1.

In the derivation data set, the median follow-up period was 56 wk (range, 4-104 wk). The CP median score was 7 (range, 5-14) with 61% of the patients being CP class B and C. The MELD and BioCliM median scores were 17 (range, 8-42) and 7.7 (range, 5.7-13.6), respectively. During follow-up, 29 patients (17%) died. The 4-, 12-, 24-, 52- and 104-wk survival rates were 98%, 98%, 90%, 89% and 83%, respectively.

The patients of the validation group were followed for a median of 58 wk (range, 8–104 wk) during which 13 died. The 4-, 12-, 24-, 52- and 104-wk survival rates were 96%, 95%, 88%, 84% and 83%, respectively. The CP median score was 7 (range, 5-14) with 65% of the patients being CP class B and C. The MELD and BioCliM median scores were 18 (range, 9-43) and 7.9 (range, 6-13.8), respectively.

None of the patients in the derivation or validation groups underwent liver transplantation during the follow-up period.

Overall survival according to single prognostic factors

Univariate analysis for 104-wk overall survival: Univariate analysis using Cox proportional hazards models showed that serum levels of creatinine, bilirubin, cholesterol, albumin, prothrombin time, partial thromboplastin time, ascites, spontaneous bacterial peritonitis, encephalopathy and bleeding esophageal varices were significantly associated with survival (Table 2).

Multivariate analysis for 104-wk overall survival: Multivariate Cox regression analysis included those variables independently related to survival resulting from univariate analysis. The selected variables were available in all patients that entered the forward stepwise model. Of the candidate variables, only ascites, encephalopathy, bleeding esophageal varices and serum creatinine were independently predictive of survival (Table 3).

Table 2 Association of baseline characteristics with mortality in 170 cirrhotic patients, results from univariate Cox proportional hazards models

Variables	*P*	Hazard ratio	95% CI for Hazard ratio
Age (yr)	0.68	0.56	0.36-1.06
Sex (male)	0.54	0.44	0.89-1.26
Etiology (viral)	0.66	0.58	0.40-1.11
ALT (IU/L) ($\log_n$ value)	0.85	0.84	0.34-1.12
AST (IU/L) ($\log_n$ value)	0.43	0.90	0.56-1.34
ALT/AST ratio	0.64	0.87	0.50-1.21
Platelet count ($\times 10^9$/L) ($\log_n$ value)	0.54	0.89	0.52-1.30
Prothrombin time (s)[1] ($\log_n$ value)	0.01	2.23	1.24-4.89
INR for prothrombin time ($\log_n$ value)	0.03	1.99	1.13-3.96
Partial thromboplastin time (s)[2] ($\log_n$ value)	0.04	1.78	1.10-3.23
Albumin (mg/dL) ($\log_n$ value)	0.001	3.12	1.89-5.23
Bilirubin (mmol/L) ($\log_n$ value)	< 0.001	3.89	2.12-6.14
Creatinine (mmol/L) ($\log_n$ value)	< 0.001	3.95	2.18-6.56
Cholesterol (mmol/L) ($\log_e$ value)	0.03	1.83	1.34-3,42
Ascites	< 0.001	4.05	2.27-6.33
Spontaneous bacterial peritonitis	0.001	3.05	2.10-5.07
Encephalopathy	< 0.001	4.50	2.90-6.50
Bleeding esophageal varices	< 0.001	4.78	3.11-7.11

CI: Confidence interval; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase. Hazard ratios (95% CI) for quantitative variables are expressed for 1 relevant unit increase of log. INR: International normalized ratio. [1]Prothrombin time (s): Value in seconds. [2]Partial thromboplastin time (s): Value in seconds.

The estimated hazard risk for ascites suggested that the risk of death for uncontrolled ascites was 10.2 times greater than for those with absent or controlled ascites. The risk of death in those patients with relapsing bleeding and uncontrolled encephalopathy increased 3.25 times compared to those without bleeding or with non-relapsing bleeding, and 2.5 times compared to those with absent or controlled encephalopathy. In terms of impact in prognosis, the ascites (hazard ratio (HR), 10.2) and serum creatinine (HR, 3.99) were the most important prognostic factors.

Model derivation and development

In the model derivation cohort, 11 potential variables selected from the univariate analysis ($P < 0.05$) were calculated for model inclusion. Of these only 5 were included in the model. The likelihood ratio statistic showed the significance of the addition of each variable separately to a predictive model that included ascites only (Table 3). The χ^2 statistic was progressively increased with the addition of creatinine, bleeding esophageal varices, hepatic encephalopathy, and bilirubin. The c-statistic in the model that included only ascites was 0.76, based on the c-statistic for censored data. When creatinine, BEV, HE and bilirubin were added to the model, the c-statistic was improved to 0.83, 0.85, 0.89, and 0.90, respectively. In the same context,

Table 3 Contributions of different variables to survival prediction at 104 wk, results from multivariate Cox regression models[1]

Variable	Variable χ^2	Regression coefficient	Hazard ratio	95% CI for Hazard ratio		*P* value	c-statistic	BIC
Ascites	53.90	2.310	10.2	3.78	28.1	< 0.0001	0.76	2014.15
+ Ln (creatinine)	63.43	1.370	3.99	1.57	10.9	0.006	0.83	1988.15
+ BEV	65.71	1.195	3.25	1.01	9.77	0.048	0.85	1970.65
+ HE	68.91	0.909	2.50	0.915	6.88	0.070	0.89	1961.89
+ Ln (bilirubin)[2]	70.11	0.349	1.46	0.66	3.33	0.427	0.90	1951.77

[1]Estimated from Cox proportional hazards models. [2]Biochemical (bilirubin and creatinine) and Clinical (ascites, encephalopathy and bleeding esophageal varices) Model; BEV: Bleeding esophageal varices; HE: Hepatic encephalopathy; BIC: Bayesian Information Criterion. Ln was used to normalize distributions and improve the fit for individual predictors. Hazard ratio for quantitative variables are expressed for 1 relevant unit increase of log. + indicates the addition of each variable separately to the model with ascites only. χ^2 is the likelihood ratio statistic for each group of variables when added to the model. The risk prediction was based on data from the model derivation cohort (n = 170) at 104 wk follow-up.

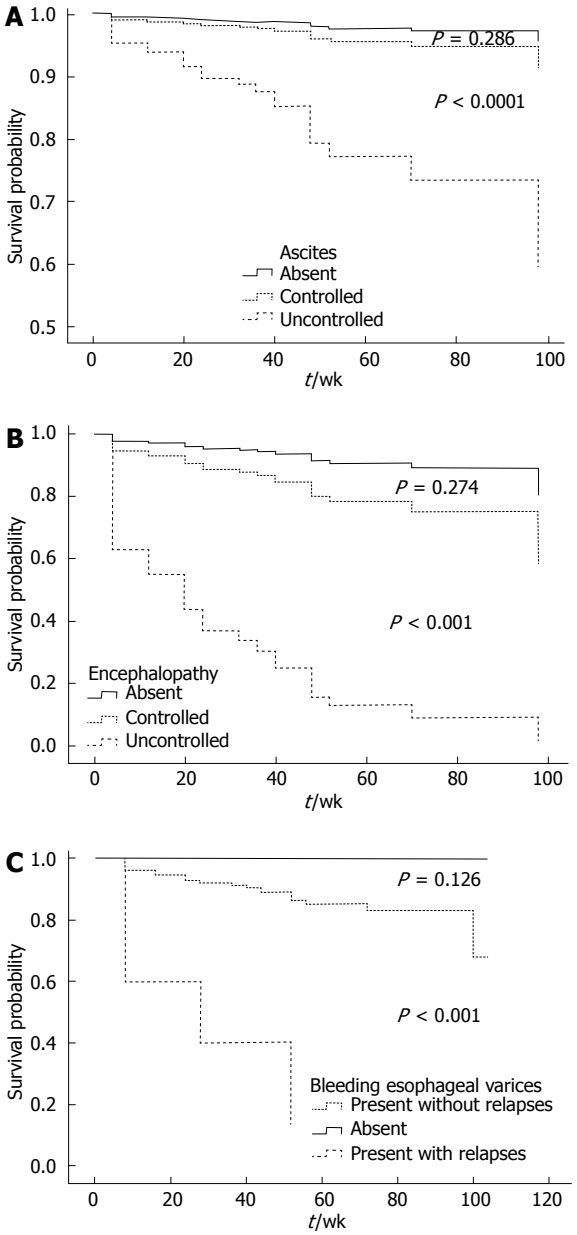

Figure 1 Kaplan-Meier estimated survival curves for clinical variables. A: Ascites; B: Encephalopathy; C: Bleeding esophageal varices.

the combination of ascites, creatinine, BEV, HE and bilirubin revealed the smallest BIC value (1951.77), thus, in the derivation set, the model with the combination of clinical and biochemical variables appeared to improve the risk prediction.

Computational formula for 104-wk risk using best-fitting model

The regression coefficients of the formula for calculating the new risk score (biochemical and clinical model) were selected from a Cox regression model[16] and are reported in Table 3.

The risk scores for individual patients were calculated using the following equation: [1.370 × $\log_e$ (creatinine mmol/L) + 0.349 × $\log_e$ (bilirubin mmol/L) + 2.310 × (ascites: 0 if absent or medically controlled and 1 if uncontrolled) + 0.909 × (encephalopathy: 0 if absent or medically controlled and 1 if uncontrolled) + 1.195 × (bleeding esophageal varices: 0 if absent or present without relapses and 1 if present with relapses). The clinical variables were coded depending on the clinical response to medical treatment. The variables grouped together as "absent or medically controlled" (ascites and encephalopathy) and "absent or present without relapses" (bleeding esophageal varices) have been so grouped because their survival was similar in each one of them (Figure 1). The missing values were imputed for survival modeling.

Survival probabilities were derived from the Cox proportional hazards model: $S(t) = S_0(t)^{\exp(R-R_o)}$. S(t) is the survival probability in wk, $S_0(t)$ the baseline survival function, R the individual risk score and R_0 the risk score of the average patient in the series. For example, the 12-wk survival probability is calculated as: $S_{(12\ wk)} = 0.981^{\exp(BioCliM\ score-7)}$, where 0.981 is the 12-wk baseline survival and 7 is the reference BioCliM score. To ease its use, the score was multiplied by 100.

Predictive models for 12-, 52- and 104-wk survival

Comparison of the c-statistic values among the CP, MELD and BioCliM scores was performed. All scoring systems were found to have diagnostic accuracy in predicting survival. The BioCliM score, however, showed to have better discriminative power in predicting short- (12 wk), intermediate- (52 wk) and long-term survival (104 wk) than the rest of the scores (Figure 2).

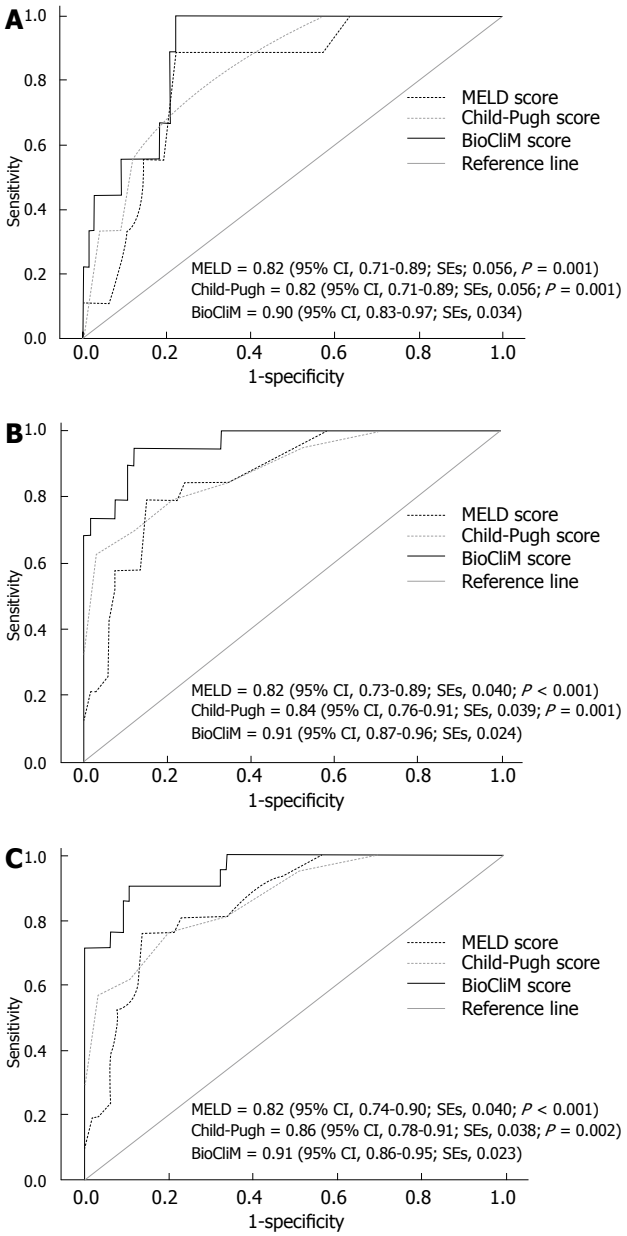

Figure 2 Comparison of the c-index values of the MELD, Child-Pugh and BioCliM scores for 12- (A), 52- (B) and 104-wk (C) survival. SE indicates standard errors. The different values were compared with BioCliM score using the bootstrap method.

The c-statistic for the CP and MELD scores were almost identical for 12-wk survival (0.82 and 0.82), and slightly higher for CP as compared with MELD for 52-wk (0.84 and 0.82) and 104-wk (0.86 and 0.82) survival.

We used an alternative way of computing the concordance probability for a censored outcome to estimate the true concordance probability in samples with a high censored proportion. The concordance probability estimates for the CP (CPE, 0.71; SE, 0.042), MELD (CPE, 0.74; SE, 0.043) and BioCliM (CPE, 0.78; SE, 0.050) models were lower at 12 wk in comparison with those obtained using the standard c-statistic value. Finally, the CPE at 12 wk was consistently higher for BioCliM as compared with CP and MELD scores.

Discrimination and model validation

The Hosmer-Lemeshow statistic (H-L) is a measure of the discrepancy between the observed and predicted risk. A better calibrated model would have a smaller discrepancy between the observed and predicted and thus a smaller H-L statistic.

A significant *P* value for the H-L statistic indicates a significant deviation between predicted and observed outcomes. Figure 3 compares the calibration of the BioCliM, MELD and CP scores in predicting the probability of death at 104 wk. The H-L statistic was 4.69 for the BioCliM score, 17.06 for the MELD score and 14.23 for the CP score, indicating a good calibration for all models; however, this analysis clearly shows that BioCliM is better calibrated.

Figure 4 illustrates the observed and expected Kaplan-Meier survival curves for each score in 2 patient subgroups divided according to risk score as low risk (R $\leqslant$ 8) and high risk (R > 8), selected from the "Calixto Garcia" Hospital. Using a cutoff value of 8 (risk score) to predict probability of survival within 104 wk, the sensitivity and specificity of the BioCliM score was 90% and 87%, respectively. Median survival was 104 wk and 47 wk for low- and high-risk groups, respectively. There were no significant differences between the observed and expected survival curves in the stratified risk groups (low risk, $P = 0.61$; high risk, $P = 0.77$). Thus, the BioCliM score allowed accurate prediction of survival in the cirrhotic patient validation group.

Survival according to the BioCliM score

The differences in the short-, intermediate- and long-term survival between patients with low risk ($\leqslant$ 8), and high risk (> 8) scores were compared (Figure 5).

Overall survival rates were significantly different between low-risk and high-risk patients ($P < 0.0001$). The 12-wk survival rates were 98% and 64% for low and high risk, respectively. For low and high risk, 1-year survival rates were 97% and 3%, and 2-year survival rates were 95% and 0%, respectively. Patients with a high risk score had the highest risk of mortality compared to patients with low values. Patients with a BioCliM score of $\geqslant$ 8 had a median survival of < 47 wk in comparison to patients with a median survival of 104 wk for patients with a BioCliM score of < 8.

DISCUSSION

The most widely used prognostic model to predict survival in cirrhotic patients has been the CP score. It is an important tool for the prognostic evaluation of cirrhotic patients and the current organ allocation policy. It has, however, several drawbacks such as the subjectivity of clinical parameters, limited discriminative capability and variability in the measurements of laboratory parameters[17,18]. Current CP score modifications by adding new variables or utilizing sophisticated measures did not improve its accuracy to predict survival[19-25]. A relatively new score, the MELD,

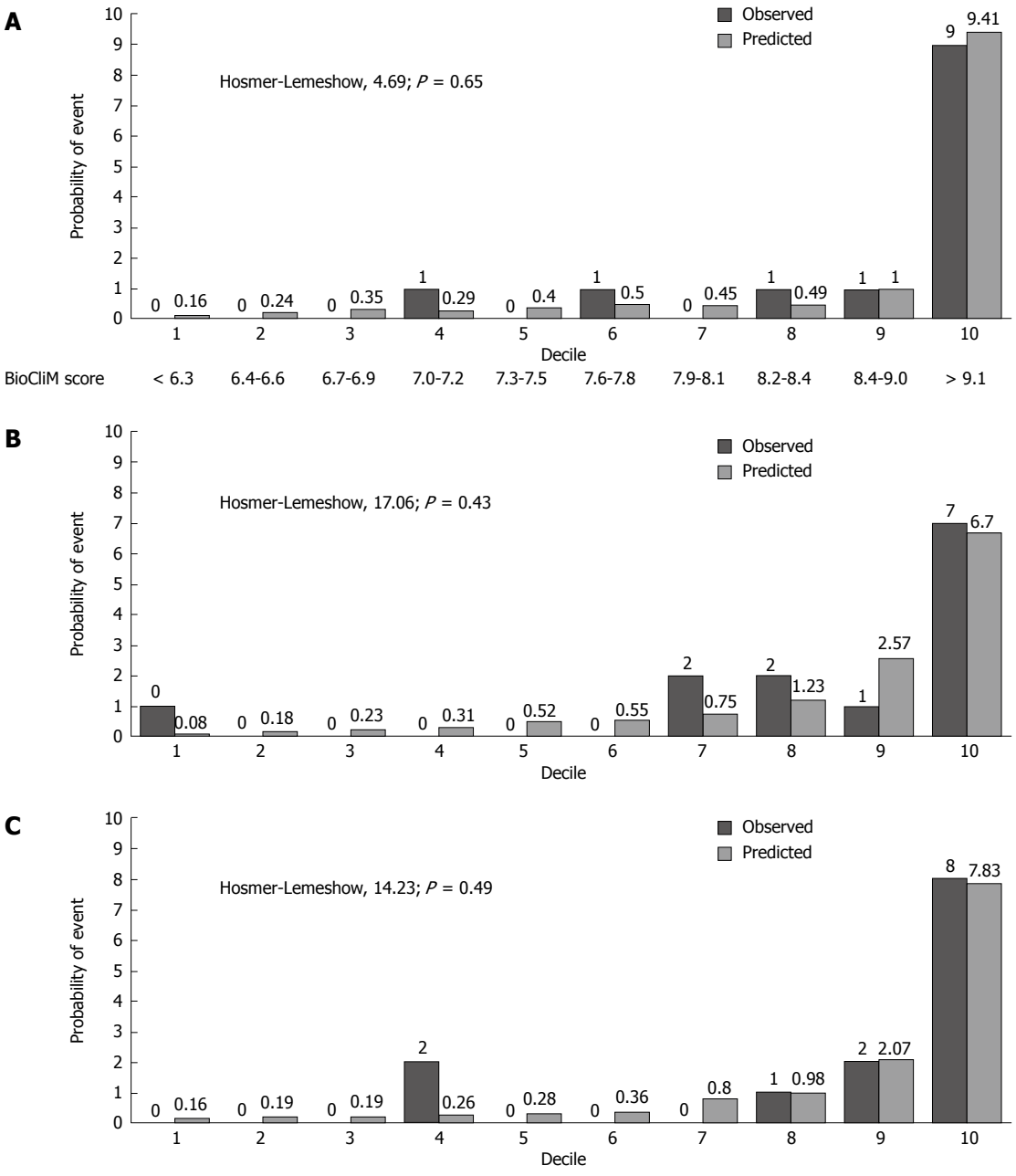

Figure 3 Observed and predicted probability of events at 104 wk. A, B and C shows the observed and predicted probability of death according to BioCliM, MELD and Child-Pugh scores in 10 groups (deciles) of patients, respectively. A significant *P*-value for the Hosmer-Lemeshow statistic indicates a significant deviation between predicted and observed outcomes.

has been instituted in patients with end-stage liver disease awaiting liver transplantation. MELD has shown an advantage over CP by using continuous objective variables that are not open to observer interpretation and are appropriately weighted according to their impact on prognosis[3,4,26]. Its ability to predict mortality, however, has been found to be similar or slightly superior to the traditional CP score[27-30]. These controversies suggest that a better predictive model is necessary to predict survival in cirrhotic patients.

In our study, the baseline characteristics were comparable with similar studies evaluating survival in cirrhotic patients[31-36]. Furthermore, all clinical and biochemical variables included in the CP and MELD scores were associated with survival in univariate analysis. Multivariate Cox proportional hazards analysis identified serum creatinine, ascites, encephalopathy and bleeding esophageal varices as independent prognostic factors for overall survival. The strongest predictors of mortality were ascites and serum creatinine. In our proposed model, ascites, encephalopathy and variceal bleeding were evaluated depending on medical treatment response, and the diagnosis and treatment of each of these was based on the most recent published guidelines[10-12,37]. The used nomenclature appeared to be more uniform and less subjective than the commonly applied classification into CP or MELD scores[2-4].

The major finding of this prospective study is that the BioCliM score, which is based on a combination of 3 clinical indices (ascites, encephalopathy and bleeding

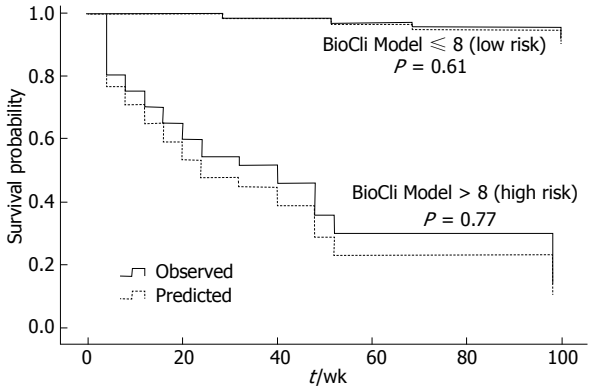

Figure 4 Observed and expected 104-wk survival curves for the BioCliM score. Survival of 85 independent patients from the "Calixto Garcia" Hospital who were stratified according to their risk score into two risk groups (low-risk ⩽ 8 and high-risk > 8). The observed and predicted BioCli Model survival curves were compared using log-rank test. The observed and expected survival was similar for the low- and high-risk groups.

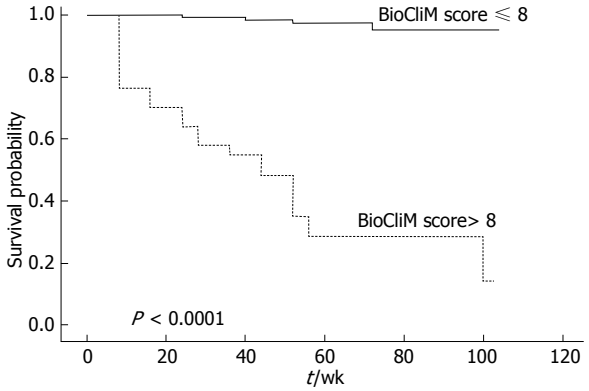

Figure 5 Kaplan-Meier estimated survival curves for the BioCli Model score.

esophageal varices) and 2 biochemical parameters (creatinine and bilirubin), is able to accurately predict short-term (12 wk), intermediate-term (52 wk) and long-term (104 wk) mortality in cirrhotic patients. Our results showed that the BioCliM score is superior to the CP and MELD scores in ranking patients according to their risk of death. In addition, the BioCliM score showed a sustained discriminative power to predict survival through the different evaluated periods (12-104 wk). Our data further support, as well as previous findings, that the MELD score is not significantly superior to the CP score in predicting survival in patients with hepatic cirrhosis[25,27-30]. Theoretically, the MELD score is undoubtedly more objective and robust than the CP score for the previously mentioned reasons; however, a major limitation of the MELD score is the poor discriminative power to predict survival among patients whose clinical course is often affected by other factors which are excluded by the model[38]. Recent studies have demonstrated that ascites, encephalopathy and hyponatremia are important independent predictors of early pretransplant mortality, especially for patients with low MELD scores[8,9,39,40], thus affecting the consideration for an expedited liver transplant under the "sickest first" model. In consequence, as the MELD score does not reflect the presence of ascites and encephalopathy, these patients need to be allocated separately for liver transplantation if MELD is used to prioritize organ allocation. By contrast, the BioCliM scale is able to accurately predict survival in patients with clinical complications of portal hypertension, thus the BioCliM score could be recommended in the individual management of these patients. Further studies are needed to validate its prognostic accuracy in patients undergoing liver transplantation.

Possibly the most important study limitations were the relatively small sample size, the poor geographic diversity of the patients included (single and tertiary center) and the major drawbacks of the MELD score related to wide variability of laboratory parameters such as serum creatinine and bilirubin[28,41,42].

In conclusion, both the CP and MELD scores can accurately predict short-term survival in cirrhotic patients, while the BioCliM score appears to have great discriminative power for short- (4 and 12 wk), intermediate- (24 and 52 wk) and long-term (104 wk) survival. In contrast to the MELD score, the use of the BioCliM score in patients with ascites, encephalopathy and variceal bleeding could significantly increase survival predictive values in patients with end-stage liver disease.

ACKNOWLEDGMENTS

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COMMENTS

Background

The Child-Pugh and Model for End-stage Liver Disease (MELD) scores are important tools for the prognostic evaluation of cirrhotic patients and the current organ allocation policy. These have, however, several drawbacks such as the subjectivity of clinical parameters, limited discriminative capability and variability in the measurements of laboratory parameters. The current evidence suggests that a better predictive model is necessary to predict survival in cirrhotic patients.

Research frontiers

The clinical complications of portal hypertension such as ascites, encephalopathy, spontaneous bacterial peritonitis (SBP) and gastrointestinal bleeding are not considered in the MELD score, probably underestimating that they may have a direct association with the severity of liver disease. The classification applied to the clinical complications of portal hypertension (ascites, encephalopathy, variceal bleeding and SBP) in the MELD score does not clearly reveal the different grades of severity of liver disease and its clinical response to medical treatment. Therefore, its utility in the prognostic model could be limited. In this study, the authors have evaluated a new paradigm for clinical variables, depending on the severity and medical treatment response and how they have an influence, as prognostic factors, in the survival of cirrhotic patients.

Innovations and breakthroughs

Recent reports have demonstrated that ascites, encephalopathy and hyponatremia are important independent predictors of early pretransplant mortality, especially for patients with low MELD scores, thus affecting the consideration for an expedited liver transplantation under the "sickest first" model. In consequence, as the MELD score does not reflect the presence of

ascites and encephalopathy, these patients need to be allocated separately for liver transplantation if MELD is used to prioritize organ allocation. By contrast, the new biochemical and clinical model is able to accurately predict survival in patients with clinical complications of portal hypertension; thus the BioCliM score could be recommended in the individual management of these patients.

Applications

In contrast to the MELD score, BioCliM is able to accurately predict survival in patients with clinical complications of portal hypertension, thus the BioCliM score could be recommended in the individual management of these patients. Further studies are needed to validate its prognostic accuracy in patients undergoing liver transplantation.

Terminology

BioCliM is a new biochemical and clinical model that is able to accurately predict survival in patients with end-stage liver disease.

Peer review

The authors examined the prognostic value and predictive capability of a new prognostic model in patients with end-stage liver disease. A less subjective nomenclature to assess the clinical complications of portal hypertension was evaluated in combination with biochemical variables to determine their influence as prognostic factors of survival in cirrhotic patients. The biochemical and clinical model was shown to accurately predict survival in patients with clinical complications of portal hypertension and it appeared to have great discriminative power for short- (4 and 12 wk), intermediate- (24 and 52 wk) and long-term (104 wk) survival.

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BRIEF ARTICLES

Association of hepatitis C virus infection and diabetes in central Tunisia

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Abstract

AIM: To investigate hepatitis C virus (HCV) seroprevalence in Tunisian patients with diabetes mellitus and in a control group.

METHODS: A cross-sectional study was conducted to determine the HCV seroprevalence in 1269 patients with diabetes (452 male, 817 female) and 1315 non-diabetic patients, attending health centers in Sousse, Tunisia. HCV screening was performed in both groups using a fourth-generation enzyme immunoassay.

RESULTS: In the diabetic group, 17 (1.3%) were found to be HCV-infected compared with eight (0.6%) in the control group, although the difference was not significant (P = 0.057). Quantitative PCR was performed in 20 patients. Eleven patients were positive and showed HCV genotype 1b in all cases.

CONCLUSION: Frequency of HCV antibodies was low in patients with diabetes and in the control group in central Tunisia, with no significant difference between the groups.

Key words: Hepatitis C virus; Diabetes mellitus; Tunisia; Epidemiology; Autoantibodies; Hepatitis

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INTRODUCTION

In recent years, a positive association between hepatitis C virus (HCV) infection and diabetes mellitus (DM) had been reported in a number of clinical studies[1-4]. It is now clear that hepatitis C conveys a risk for developing DM, in particular type 2[5-7]. Moreover, several studies have found a high prevalence of anti-HCV antibodies among patients with diabetes, especially those with type 2 DM[8-13]; however, some authors have not observed an association between HCV infection and diabetes[14-16]. Since effective therapy has become available for HCV, it may be worthwhile to determine virus prevalence in patients with and without diabetes, in order to decide whether a programme for screening should also focus on type 2 diabetes. The aim of the present study was to investigate HCV seroprevalence in Tunisian patients with DM and in a control group.

MATERIALS AND METHODS

Patients

During March 2003, we conducted a cross-sectional study of all consecutive patients with diabetes aged > 16 years who were attending the Departments of Internal Medicine, Infectious Diseases and Endocrinology of Farhat Hached Hospital, and primary health care centers in the region of Sousse, Tunisia. Sample size calculation was

based on a 2% HCV seroprevalence estimation with an 80% precision rate and a 95% confidence level. The formula for sample size determination yielded a total of 1223 patients with diabetes. Types 1 and 2 diabetes were defined on the basis of a history of therapy with oral hypoglycemic agents or insulin at the date of inclusion. Patients older than 40 years of age, and treated by oral hypoglycemic agents or switched from insulin were considered to have type 2 diabetes. A control group of non-diabetic patients were recruited from the same centers at the same time. Patients who had corticosteroid-induced diabetes were excluded. Informed consent was obtained from all participants, and the study was approved by Farhat Hached Hospital ethics committee.

Data collection

Data were recorded by using a questionnaire that collected information on demographic and clinical features of DM and risk factors for HCV infection. Blood samples were collected from all patients for HCV serology. Those who were positive for anti-HCV antibodies were called, and liver function tests, glucose blood level, HCV quantitative RNA and HCV genotyping were performed.

Laboratory methods

Serological testing for anti-HCV antibodies was performed by using a fourth-generation ELISA (Murex; Abbot Laboratories, France) according to the manufacturer's instructions. HCV RNA qualitative and quantitative testing (Amplicor; Roche Molecular Systems, Branchburg, NJ, USA) and HVC genotyping were performed at Pasteur Cerba Laboratoire, Cergy Pontoise, France). HVC genotyping was performed by RT-PCR on a segment from the core region and by hybridization of this fragment with oligonucleotide-specific probes. The assay was designed to recognize the 1a, 1b, 2a, 2b, 3, 4, 5 and 6 HCV genotypes.

Statistical analysis

Data were analyzed, using SPSS version 13.0 software (Chicago, IL, USA). A descriptive analysis was followed by bivariate analysis using the χ^2 test for comparison of the two groups, with a 5% statistical significance level. A multivariate analysis with logistic regression was used to determine predictive variables associated with seroprevalence among the significant factors found by bivariate analysis. ORs and 95% CI were calculated for these variables.

RESULTS

Our study included 1269 patients with diabetes and 1315 non-diabetic patients. In patients with diabetes, 1148 (90.5%) and 121 (9.5%) had type 2 and type 1 DM respectively; 284 (22.5%) were treated by insulin. The mean duration of DM was 8.4 years (1-35 years). Furthermore, history of surgery and hospitalization, and scarification were found to be more frequent in patients with diabetes. Patients in the control group were much younger than those with diabetes; the main demographic and clinical characteristics of both groups are shown in Table 1.

Table 1 Epidemiological features of the study population in patients with diabetes and control group *n* (%)

	Diabetes patients (*n* = 1269)	Control group (*n* = 1315)	*P*
Gender			
Female	817 (64.4)	890 (67.6)	0.06
Age			
Mean age (yr)	55.6	46.9	$< 10^{-3}$
Risk factors of HCV			
Transfusion	157 (12.5)	185 (14.2)	0.2
History of surgery	592 (46.8)	551 (42.1)	0.01
Drug addiction	2 (0.2)	7 (0.5)	0.17
Scarification	247 (19.8)	301 (23.1)	0.04
Endoscopic investigation	276 (21.9)	308(23.6)	0.30
Alcoholism	164 (13)	178 (13.6)	0.65
History of hospitalization	875 (69.3)	785 (59.9)	$< 10^{-3}$
Anti-HCV antibodies (+)	17 (1.3)	8 (0.6)	0.057

Table 2 Epidemiological features of the study population in type 2 DM patients and control group *n* (%)

	Type 2 DM patients (*n* = 1148)	Control group (*n* = 1315)	OR	*P*
Gender				
Female	739 (65.2)	890 (67.6)	0.8 (0.73-1.2)	0.06
Age				
Mean age (yr)	57 ± 10.4	46.9		$< 10^{-3}$
Risk factors of HCV				
Transfusion	136 (12.1)	185 (14.2)	0.83 (0.65-1.06)	0.2
History of surgery	528 (46.7)	551 (42.1)	1.18 (1.05-1.39)	0.01
Drug addiction	2 (0.2)	7 (0.5)	0.33 (0.05-1.78)	0.17
Scarification	222 (19.8)	301 (23.1)	0.81 (0.66-0.99)	0.04
Endoscopic investigation	248 (22)	308 (23.6)	0.90 (0.74-1.09)	0.30
Alcoholism	144 (12.8)	178 (13.6)	0.92 (0.78-1.17)	0.65
History of hospitalization	758 (67.1)	785 (59.9)	1.31 (1.11-1.55)	$< 10^{-3}$
Anti-HCV antibodies (+)	16 (1.4)	8 (0.6)	2.31 (1.01-5.90)	0.04

Antibodies against HCV were detected in 25 patients (1%) among the entire population studied (both diabetic and non-diabetic groups). In the diabetes group, 17 (1.3%) were found to be infected with HCV compared with eight (0.6%) control patients. No significant difference was found between DM patients and the control group ($P = 0.057$) (Table 1). Moreover, anti-HCV seropositivity was detected in 16 (1.4%) of the type 2 DM sub-group, which was significantly higher than that in the control group ($P = 0.04$) (Table 2). However, in multivariate analysis, this difference between seroprevalence of HCV in type2 DM and controls was not confirmed.

Quantitative PCR was performed in 20 patients: 13 with diabetes and seven without, was and a positive result

was obtained in eight and three patients, respectively. All patients were infected by genotype 1b HCV. All non-diabetic patients who were positive for HCV antibodies underwent liver function and blood glucose testing, but no new DM was discovered.

DISCUSSION

To the best of our knowledge, this is the first study in which HCV infection prevalence was evaluated in Tunisian patients with diabetes. Similar to blood donors in whom anti-HCV antibodies were low (0.5%-1.8%)[17-21], in our study HCV infection prevalence in the diabetes, type 2 DM, and control groups was 1.3%, 1.4% and 0.6%, respectively. Despite a high frequency of scarification, history of surgery and hospitalization in patients with diabetes and the type 2 DM subgroup, circumstances which increase risk of HCV infection, prevalence of anti-HCV antibodies was not significantly more frequent in the diabetes group ($P = 0.057$). Moreover, comparing type 2 DM patients to the control group, although a significant difference in HCV infection prevalence was observed in type 2 DM patients ($P = 0.04$), this was not confirmed by logistic regression analysis. Therefore, we cannot establish the diabetic population as a group at high risk for HCV infection. Our findings did not confirm other studies that have reported increased HCV seroprevalence in patients with diabetes[10,22-26]. In a case-control study conducted in the USA, 4.2% of 594 patients in a cohort with diabetes were found to be infected with HCV compared with 1.6% of control patients (377 patients with thyroid diseases)[27]. Other studies have reported an increased HCV seroprevalence, varying from 8% to 11% in European diabetic populations compared with 1%-2% HCV seroprevalence in the general population[10,28-30]. However, in a descriptive Greek study of patients with diabetes without a control group, HCV antibodies were detected in only seven cases, and this prevalence (1.65%) was similar to that in the general population[14].

In conclusion, our study confirms a low prevalence of anti-HCV antibodies in Tunisian patients with diabetes, and may argue against diabetes as a risk factor of HCV infection in this area. Further studies, possibly multicenter, prospective and case-control, are needed to establish the temporal relationship between HCV infection and DM.

COMMENTS

Background

Several studies have found a high prevalence of anti-hepatitis C virus (HCV) antibodies among patients with diabetes mellitus (DM), especially those with type 2 DM. However, some authors have not observed an association between HCV infection and diabetes. Since effective therapy has become available for HCV, it may be worthwhile to determine the prevalence of HCV in patients with and without diabetes, to decide whether a programme for screening should also focus on type 2 diabetes patients.

Research frontiers

The literature is still contradictory about high prevalence of HCV infection in type 2 DM. The prevalence of HCV infection is still unknown in Tunisia. In this study, the authors demonstrated that this prevalence was similar in the general population.

Innovations and breakthroughs

The study confirmed a low prevalence of anti-HCV antibodies in Tunisian patients with diabetes, and may disprove diabetes as a risk factor for HCV infection in this area.

Applications

The low prevalence of HCV infection in type 2 DM in this study argues against the systematic assessment of HCV antibodies in this population.

Peer review

The present manuscript describes a comparative analysis of HCV prevalence in diabetic and non-diabetic populations in central Tunisia. Although its findings are negative, they are interesting because of the relatively large sample size. This is an interesting small epidemiological study on the association between diabetes and HCV prevalence in central Tunisia.

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BRIEF ARTICLES

A dose-up of ursodeoxycholic acid decreases transaminases in hepatitis C patients

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Abstract

AIM: To examine whether a dose-up to 900 mg of ursodeoxycholic acid (UDCA) decreases transaminases in hepatitis C patients.

METHODS: From January to December 2007, patients with chronic hepatitis C or compensated liver cirrhosis with hepatitis C virus (HCV) (43-80 years old) showing positive serum HCV-RNA who had already taken 600 mg/d of UDCA were recruited into this study. Blood parameters were examined at 4, 8 and 24 wk after increasing the dose of oral UDCA from 600 to 900 mg/d.

RESULTS: Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and gamma-glutamyl transpeptidase (GGT) levels were significantly decreased following the administration of 900 mg/d as compared to 600 mg/d. The decrease in ALT from immediately before the dose-up of UDCA to 8 wk after the dose-up was 14.3 IU/L, while that for AST was 10.5 IU/L and for GGT was 9.8 IU/L. Platelet count tended to increase after the dose-up of UDCA, although it did not show a statistically significant level (P = 0.05). Minor adverse events were observed in 3 cases, although no drop-outs from the study occurred.

CONCLUSION: Oral administration of 900 mg/d of UDCA was more effective than 600 mg/d for reducing ALT, AST, and GGT levels in patients with HCV-related chronic liver disease.

Key words: Chronic hepatitis; Hepatitis C virus; Liver fibrosis; Transaminase; Ursodeoxycholic acid

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INTRODUCTION

Current treatment for chronic hepatitis C virus (HCV) infection is based on the administration of pegylated interferon (IFN) alone or in combination with other anti-viral agents such as ribavirin or protease inhibitors. However, these treatments are not completely effective in all patients with HCV genotype 1 and high viral load or in patients with liver cirrhosis[1-4]. Ursodeoxycholic acid (UDCA) was identified in 1902 from polar bear bile by Hammarsten and was isolated and crystallized by Shoda[5]. UDCA is used worldwide for the treatment of primary biliary cirrhosis (PBC) and chronic liver diseases[6-14]. Up to 2006, a dose of 150 mg/d of UDCA was approved as the standard treatment for hepatic protection in patients with chronic viral hepatitis by the public health insurance agency of Japan. However, this dosage is not effective for the treatment of chronic hepatitis[15]. A randomized, controlled-dose study of UDCA for chronic hepatitis C (CH-C) patients reported that UDCA administered at a dose of 600 or 900 mg/d resulted in greater decreases in the serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and gamma-glutamyl

transpeptidase (GGT) compared to 150 mg/d, which was the dose recommended by the Japanese national health insurance policy at that time; however, the results with doses of 600 mg/d or 900 mg/d were similar. In contrast, 600 mg of UDCA, which is the maximum administration dose in PBC or other biliary system diseases such as gallstones, was used ambiguously in CH-C patients[16].

To determine the effect of 900 mg/d of UDCA for CH-C, the present study was conducted primarily as a dose-up trial from 600 mg/d to 900 mg/d in hepatitis C patients, with changes in ALT levels as the primary endpoint.

MATERIALS AND METHODS

Patients

From January to December in 2007, patients with CH-C or compensated liver cirrhosis with HCV (mean age 65.8, range 43 to 80 years) who tested positive for serum HCV-RNA were recruited into this study. All the enrolled patients had already received 600 mg/d of UDCA, and showed over 40 IU/L of ALT at the two points in the 4 wk prior to dose-up of UDCA. Patients were excluded from the study if they had received antiviral treatment with interferon with or without ribavirin or anticancer treatment for hepatocellular carcinoma (HCC). Patients with other malignancies diagnosed within 24 wk before the observation period or patients treated with corticosteroids and/or immunosuppressive drugs were also excluded. Patients with decompensated cirrhosis, hepatitis B, autoimmune liver disease, alcoholic or drug-induced liver injury, malignant tumors and biliary disorders were excluded. Patients receiving intravenous glycyrrhizin were enrolled in this study. However, when the dose or frequency of administration of glycyrrhizin was changed, this was defined as the study endpoint. Written informed consent was obtained from each patient before enrollment into the study.

Methods

After the 4-wk observation period, the dose of UDCA (Urso®, Mitsubishi Tanabe Pharma Corp., Osaka, Japan) was increased from 600 mg/d to 900 mg/d. Serum ALT was measured as a primary endpoint of liver function, and AST and GGT as secondary endpoints, using conventional methods. Blood samples were taken at the start of the observation period, at 0, 4, 8 and 24 wk after initiation of treatment, and at the final observation period. Serum concentrations of ALT, AST, GGT, albumin, total bilirubin and platelet counts were measured. CT and ultrasonography for HCC screening was carried out every 12 wk or 24 wk. Compliance with UDCA administration and adverse effects were determined by patient interview or confirmation of drug diaries.

Statistical analysis

Changes in AST, ALT, GGT, total bilirubin, albumin, and platelet count were analyzed by paired Student's *t*-test. $P < 0.05$ was considered significant.

Table 1 Patient characteristics before beginning the study (mean ± SE)

	Total (n = 32)
Mean age (range)	65.8 ± 2.6 (43-80)
Gender (male)	18 (56)
Liver cirrhosis (%)	7 (22)
Controlled hepatocellular carcinoma (%)	4 (12)
Glycyrrhizin administration (%)	6 (19)
AST (IU/L)	66.5 ± 4.1
ALT (IU/L)	57.1 ± 3.4
GGT (IU/L)	44.2 ± 2.1
Total bilirubin (mg/dL)	0.76 ± 0.5
Serum albumin (g/dL)	4.0 ± 0.1
Platelet count (× 1000/μL)	14.5 ± 1.0
HCVRNA (KIU/mL)	1309 ± 469
HCV genotype (1b/non 1b/not decided)	24/6/2

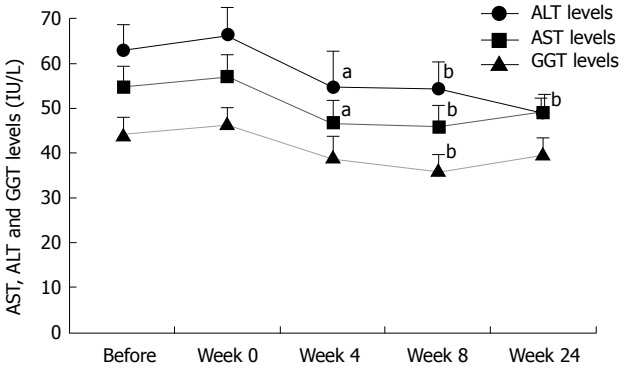

Figure 1 Changes in serum alanine aminotransferase (ALT) levels, serum aspartate aminotransferase (AST) levels and serum gamma-glutamyl transpeptidase (GGT) levels in patients before and during dose-up to 900 mg/d. Data are expressed as mean ± SD. $^aP < 0.05$, $^bP < 0.01$; paired *t*-test compared to week 0 in each parameter.

RESULTS

We enrolled 32 patients to this study. Patient characteristics are described in Table 1. In seven patients with liver cirrhosis, five patients were estimated as Child A and the others as Child B. Three patients with a history of HCC had been clinically diagnosed by dynamic computed tomography as having a complete response to trans-catheter arterial embolization and/or percutaneous radiofrequency ablation 24 wk or more before the start of the observation period. Compliance rate with UDCA administration was over 95%.

Changes in AST, ALT and GGT by dose-up of UDCA

Serum ALT, AST and GGT levels before and after the start of 900 mg of UDCA are shown in Figure 1. Serum ALT, AST and GGT levels were significantly decreased at 4, 8 and 24 wk after dose-up to 900 mg/d. The decrease (decreasing rate, %) in ALT levels before and 8 wk after dose-up to 900 mg of UDCA was 14.3 IU/L (22.1%) as shown in Figure 1. The decrease in AST and GGT were 10.5 IU/L (19.1%), and 9.8 IU/L (22.1%), respectively (Figure 1).

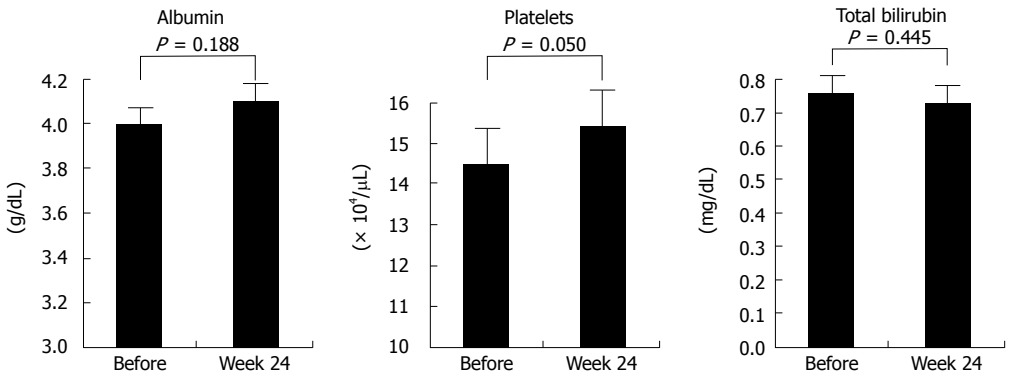

Figure 2 Changes in serum albumin, platelet count, and total bilirubin levels in chronic hepatitis C patients before and 24 wk after beginning the dose-up to 900 mg/d.

Changes in serum concentrations of albumin, total bilirubin and platelet count

Serum albumin level changed from 4.0 g/dL to 4.1 g/dL at 24 wk after the dose-up of UDCA. Platelet count changed from 145 000 to 154 000/μL, and total bilirubin changed from 0.76 to 0.73 mg/dL, although the difference did not reach a statistically significant level (P = 0.05, Figure 2). Serum HCV-RNA level did not change during the study period.

Safety

The number of adverse events during the administration of 900 mg UDCA, totaled three (9.4%), mild diarrhea in two patients and mouth discomfort in one patient. None of these adverse events influenced compliance with UDCA. Although HCC recurrence was detected in one patient at just 24 wk after dose-up of UDCA, this lesion was completely treated with percutaneous radiofrequency ablation.

DISCUSSION

The results of this study revealed that the dose-up trial of UDCA from 600 mg/d to 900 mg/d improved biochemical markers such as serum AST, ALT and GGT as early as the first or second dose-up week and continued to improve biochemical markers up to 24 wk after dose-up of UDCA was initiated. In addition, platelet count tended to increase following this dose-up therapy. These results suggested that 900 mg of UDCA can improve liver function tests in patients with chronic hepatitis C who have already received 600 mg of UDCA. In this study, the frequency of adverse events was lower than those in previous reports[15-18]. A possible reason for this is that patients enrolled in this study were not naïve to UDCA and may have quickly gotten used to the administration of UDCA.

In the natural course of CH-C, patients with normal serum aminotransferase levels show a slow fibrosis progression and a low incidence of HCC. Rino *et al*[19] demonstrated that the mode of reduction therapy and ALT levels were the most important factors, by multivariate analysis, to affect HCC development in patients with HCV-related cirrhosis of Child A classification followed for over 10 years. In addition, a previous study of postoperative patients with HCC found that recurrence was more frequent among patients with high serum ALT levels over 80 IU/L[20]. Moreover, using multivariate analysis in the Inhibition of Hepatocarcinogenesis by Interferon Therapy (IHIT) study, the risk of HCC after interferon treatment without virological response was strongly influenced by ALT levels, and the odds ratio of HCC in sustained virological responders was the same as that in sustained biochemical responders[21]. Therefore, high dose UDCA possibly reduced the occurrence and recurrence of HCC through the reduction of serum ALT level.

The anti-inflammatory mechanism of UDCA was considered to cause a reduction in the cytotoxicity of hydrophobic bile acids, stimulation of hepatobiliary secretion, suppression of NF-kB-dependent transcription by binding to the glucocorticoid receptor, and a decrease in proinflammatory cytokine-induced transcription of phospholipase A2[22-28].

The long-term effects of UDCA therapy in CH-C patients have not been fully elucidated[29]. Changes in liver histology following UDCA administration may not be clear from short-term observation periods. In this study, the dose-up treatment with 900 mg/d UDCA for 24 wk tended to increase serum platelet counts. In patients with hepatitis C virus-related chronic liver diseases, platelet counts reflect histological findings. When the platelet count is low in the patient, progression of liver fibrosis is suggested[30-33]. It is necessary to show histologically the morphological hepatic tissue changes in future studies.

In conclusion, oral administration of high dose 900 mg UDCA, despite the absence of an anti-viral effect, shows beneficial effects in reducing the activity of chronic hepatitis or cirrhosis.

COMMENTS

Background

Administration of pegylated interferon alone or in combination with anti-viral agents has improved the treatment for chronic hepatitis C, but is not very effective in some patients-especially those with hepatitis C virus (HCV) genotype 1 and high viral load or liver cirrhosis. Such patients may benefit from therapies which reduce liver inflammation and fibrosis.

Research frontiers

Researchers assessed the effect of ursodeoxycholic acid (UDCA) on serum liver enzyme levels in patients with chronic hepatitis C or compensated liver cirrhosis with HCV. Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and gamma-glutamyl transpeptidase (GGT) levels were significantly decreased with 900 mg/d compared with 600 mg/d.

Innovations and breakthroughs

Increasing the oral UDCA dose to 900 mg/d was effective in reducing ALT, AST, and GGT levels. Adverse effects were reported in 3 cases (9.4%), but none of these adverse effects influenced UDCA compliance.

Applications

The study results suggest that patients with HCV genotype 1 and high viral load or liver cirrhosis may benefit from oral UDCA therapy.

Peer review

This manuscript demonstrates that raising the UDCA dose from 600 mg/d to 900 mg/d improves liver chemistries including AST, ALT, and GGT over a 6 mo period. The authors then suggest that this may lead to suppression of fibrosis in a HCV population. Also, this research suggests that UDCA is safe at this dose. This is novel in a Japanese HCV cohort and the methods are quite straightforward. While not particularly novel, it does suggest that higher dose UDCA can improve liver chemistries.

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Spatial distribution patterns of anorectal atresia/stenosis in China: Use of two-dimensional graph-theoretical clustering

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Abstract

AIM: To investigate the spatial distribution patterns of anorectal atresia/stenosis in China.

METHODS: Data were collected from the Chinese Birth Defects Monitoring Network (CBDMN), a hospital-based congenital malformations registry system. All fetuses more than 28 wk of gestation and neonates up to 7 d of age in hospitals within the monitoring sites of the CBDMN were monitored from 2001 to 2005. Two-dimensional graph-theoretical clustering was used to divide monitoring sites of the CBDMN into different clusters according to the average incidences of anorectal atresia/stenosis in the different monitoring sites.

RESULTS: The overall average incidence of anorectal atresia/stenosis in China was 3.17 per 10000 from 2001 to 2005. The areas with the highest average incidences of anorectal atresia/stenosis were almost always focused in Eastern China. The monitoring sites were grouped into 6 clusters of areas. Cluster 1 comprised the monitoring sites in Heilongjiang Province, Jilin Province, and Liaoning Province; Cluster 2 was composed of those in Fujian Province, Guangdong Province, Hainan Province, Guangxi Zhuang Autonomous Region, south Hunan Province, and south Jiangxi Province; Cluster 3 consisted of those in Beijing Municipal City, Tianjin Municipal City, Hebei Province, Shandong Province, north Jiangsu Province, and north Anhui Province; Cluster 4 was made up of those in Zhejiang Province, Shanghai Municipal City, south Anhui Province, south Jiangsu Province, north Hunan Province, north Jiangxi Province, Hubei Province, Henan Province, Shanxi Province and Inner Mongolia Autonomous Region; Cluster 5 consisted of those in Ningxia Hui Autonomous Region, Gansu Province and Qinghai Province; and Cluster 6 included those in Shaanxi Province, Sichuan Province, Chongqing Municipal City, Yunnan Province, Guizhou Province, Xinjiang Uygur Autonomous Province and Tibet Autonomous Region.

CONCLUSION: The findings in this research allow the display of the spatial distribution patterns of anorectal atresia/stenosis in China. These will have important guiding significance for further analysis of relevant environmental factors regarding anorectal atresia/stenosis and for achieving regional monitoring for anorectal atresia/stenosis.

Key words: Spatial distribution; Anorectal atresia/stenosis; Two-dimensional graph-theoretical clustering; Incidence; Monitoring

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INTRODUCTION

Anorectal atresia/stenosis is a congenital malformation characterized by absence of continuity of the anorectal canal or of communication between rectum and anus, or narrowing of anal canal, with or without fistula, to neighboring organs[1]. Its incidence is rated high amongst gastrointestinal tract malformations. Incidence relates not only to genetic factors but also to environmental factors, especially spatial differences. There is, however, very little information available in literature about the spatial distribution patterns of anorectal atresia/stenosis in China.

Since 1986 China has been using the hospital-based Chinese Birth Defects Monitoring Network (CBDMN) to dynamically monitor severe congenital malformations such as anorectal atresia/stenosis[2]. We conducted this research to divide monitoring sites of the CBDMN into different clusters using two-dimensional graph-theoretical clustering analysis of the incidences of anorectal atresia/stenosis. Consideration was given to the similarities of the incidences of anorectal atresia/stenosis and the adjacent spatial relationships among different monitoring sites. This paper will present the spatial distribution patterns of anorectal atresia/stenosis and hopes to provide clues for research on its etiology.

MATERIALS AND METHODS

Objects

Research subjects were all perinatal fetal births more than 28 wk of gestation and neonates up to 7 d of age monitored in hospitals in the monitoring sites of the CBDMN from 2001 to 2005. They included live births, fetal deaths, stillbirths and those neonates who died within the first 7 d in these hospitals.

Monitoring hospitals

Using the hospital-based guidelines for monitoring birth defects in developing countries, as recommended by World Health Organization (WHO), the CBDMN gathered data from about 460 hospitals in this hospital-based network. These hospitals - all of them above the county level - were located in 138 cities (138 monitoring sites) of 31 different provinces, municipal cities, and autonomous regions in China. The selection of monitoring sites used the method of stratified sampling based on the combination of geographical location, economic development level and infant mortality rate. The spatial distribution of monitoring sites is in accordance with the distribution of nationwide births. The nationwide program covers approximately 450 000-500 000 births annually through all monitoring hospitals.

Information collection

The monitoring staff all received technical training on the case ascertainment of birth defects and the reporting of register forms. The monitoring hospitals collected the basic monthly information about the fetuses and neonates from units of delivery, pediatric and pathology quarterly reports and filled in the "Quarterly Form for Perinatal Births". The monitoring staff in these hospitals filled in the "Registration Card for Births with Congenital Malformations" regarding the cases of diagnosed anorectal atresia/stenosis. All the forms were required to be handed over to the provincial birth defects monitoring offices; these would be reported to the National Center for Birth Defects Monitoring after scrutiny. The specific monitoring methods and quality control measures complied with those in reference[3].

Inclusion and exclusion criteria

The perinatal births diagnosed as having anorectal atresia/stenosis with reference to criteria in Code Q42.1 and Code 42.3 in ICD-10 were included in this research. According to the criteria authorized by the International Clearing house for Birth Defects Surveillance and Research (ICBDSR), cases of mild stenosis which did not need correction and ectopic anus were excluded.

Spatial distribution analysis

The Excel Package was used to build the database of data of anorectal atresia/stenosis by monitoring sites. The ArcView GIS 3.2 was applied to spatially display the average incidences of anorectal atresia/stenosis in different provinces, municipal cities, and autonomous regions.

Two-dimensional graph-theoretical clustering: The graph is a set of vertices and edges that connect pairs of vertices in the space[4-6]. According to the basic requirements for clustering the two-dimensional ordinal samples, the similarities of the disease-related variables between members of the same cluster and their disparities between members of different clusters need to be maintained. The connectivity of the geographic units within the cluster also needs to be conserved. The weighted connected graph was supposed to be G = (V, E, D), in which (1) V represents the set of the locations of the geographic units (referred to monitoring sites in this research), (2) E represents the initial location connection matrix $B^{(0)}$ (Formula 1), the set of the adjacent relationships among different monitoring sites, and (3) D represents the initial disease-related distance matrix $D^{(0)}$ (Formula 2), the weights between different vertices in the tree algorithm in the graph theory. Based on the weighted connected graph G = (V, E, D), minimum spanning trees (MST) which were of biogeographic significance[7,8] were constructed by the Kruskal MST algorithm[9]. The two vertices with the minimum distance measures were selected and connected. One of the remaining vertices was selected and connected with the one of the two connected vertices to which it showed the minimum distance measured. The other remaining vertices were connected consecutively with those vertices already connected in the same manner until all the vertices were interconnected. The whole process was completed by the DPS7.05 software package[10].

$B^{(0)} = (b_{ik}^{(0)})_{n \times n}$; $i, k = 1, 2, ..., n$ (Formula 1)

$D^{(0)} = (d_{ik}^{(0)})_{n \times n}$; $i, k = 1, 2, ..., n$ (Formula 2)

Where $b_{ik}^{(0)}$ is the labeling of location connection between the monitoring site i and the monitoring site k. The value of $b_{ik}^{(0)}$ is 1 if the two monitoring sites are adjacent, while it is 0 when the two monitoring sites are not. $d_{ik}^{(0)}$ is the similarity distance between the incidence of the monitoring site i and that of the monitoring site k.

The MST was deconstructed by the method of "necks" in the graph theory[11]. Specific steps processed were as follows: (1) calculating the "branches": All n vertices were interconnected by (n-1) edges. Two of these vertices were connected only by one edge and the others were connected by at least two edges, which thus formed a chain without circuits, called the "branch". The branch with the most edges was called the main branch (or diameter) of the MST; (2) calculating the "subsidiary main branch". Starting from any vertex in the main branch of MST, the branch with the most edges, other than the main branch, was separated out and called the subsidiary main branch. The number of edges of the subsidiary main branch was called the "depth" of the vertex; (3) identifying "necks". The task was twofold: (Ⅰ) to appoint an integer "a" (that is > 1), and (Ⅱ) to find the subsidiary main branch of every vertex with a depth ≥ a in the main branch. The edges, which connected the vertices with the depth of 0 in the main shared parts of every subsidiary main branch, were called the "neck"; and (4) the necks were deleted in the graph to deconstruct the MST into parts so that the monitoring sites in the graph were divided into different clusters accordingly.

The layer of the deconstructed MST was added to the Administrative Boundary Layer of the 1:4M-scale Topographic Database of the National Fundamental Geographic Information System of China to formulate the two-dimensional MST-based cluster graph. The clustering results were used to make another cluster map for visual observation. This process was performed by the ArcView GIS 3.2 package software.

RESULTS

A total of 2670367 perinatal births were monitored from 2001 to 2005 all over China. Eight hundred and forty six cases of anorectal atresia/stenosis were found, equating to a total average incidence of 3.17 per 10000. See Table 1 for the average incidences of anorectal atresia/stenosis in different provinces, municipal cities or autonomous regions. The top five incidences appeared in Liaoning (4.89 per 10000 births), Zhejiang (4.83 per 10000 births), Guangdong (4.78 per 10000 births), Chongqing (4.59 per 10000 births) and Beijing (4.10 per 10000 births).

Spatial distribution

Regarding the geographic division standard for Eastern, Middle and Western China from the National Bureau of Statistics of China in 2003[12], the areas (provinces, autonomous regions, or municipal cities) with the highest average incidences of anorectal atresia/stenosis were concentrated in Eastern China, while the areas with the lowest average incidence of less than 1.99 per 10000 were mostly located in Western China (Figure 1).

Table 1 Average incidences of anorectal atresia/stenosis in different provinces, municipal cities or autonomous regions of China from 2001 to 2005

Province/Autonomous region/Municipal city	Perinatal births	Cases	Average incidence (per 10000)
Liaoning	79760	39	4.89
Zhejiang	111690	54	4.83
Guangdong	127648	61	4.78
Chongqing	60979	28	4.59
Beijing	114741	47	4.10
Guangxi	64444	26	4.03
Tianjin	62542	25	4.00
Anhui	100631	36	3.58
Fujian	95970	34	3.54
Ningxia	65064	23	3.53
Jiangsu	149984	52	3.47
Jilin	112606	38	3.37
Shanxi	76653	24	3.13
Henan	126082	39	3.09
Hubei	59964	18	3.00
Hebei	130441	39	2.99
Gansu	61629	18	2.92
Hunan	85919	25	2.91
Heilongjiang	71224	20	2.81
Hainan	54234	14	2.58
Shandong	164141	42	2.56
Guizhou	58695	15	2.56
Sichuan	88783	22	2.48
Shanghai	144361	34	2.36
Inner Mongolia	63061	14	2.22
Yunnan	85398	17	1.99
Jiangxi	84015	15	1.79
Xinjiang	56214	10	1.78
Shaanxi	64634	11	1.70
Qinghai	36666	6	1.64
Tibet	12194	0	0.00
Total	2670367	846	3.17

Results of the two-dimensional graph-theoretical clustering

The MST was constructed with consideration to the similarities of average incidences of anorectal atresia/stenosis and the spatial connectivity between different monitoring sites (Figure 2). According to the "neck" calculation method in the graph theory, when the integral constant, a, was designated as 2, the monitoring sites were divided into 6 clusters of different areas.

Regarding the average incidences of anorectal atresia/stenosis in different provinces, municipal cities and autonomous regions from 2001 to 2005, the monitoring sites were grouped into 6 clusters. Cluster 1 comprised the monitoring sites in Heilongjiang Province, Jilin Province, and Liaoning Province; Cluster 2 was composed of those in Fujian Province, Guangdong Province, Hainan Province, Guangxi Zhuang Autonomous Region, south Hunan Province,

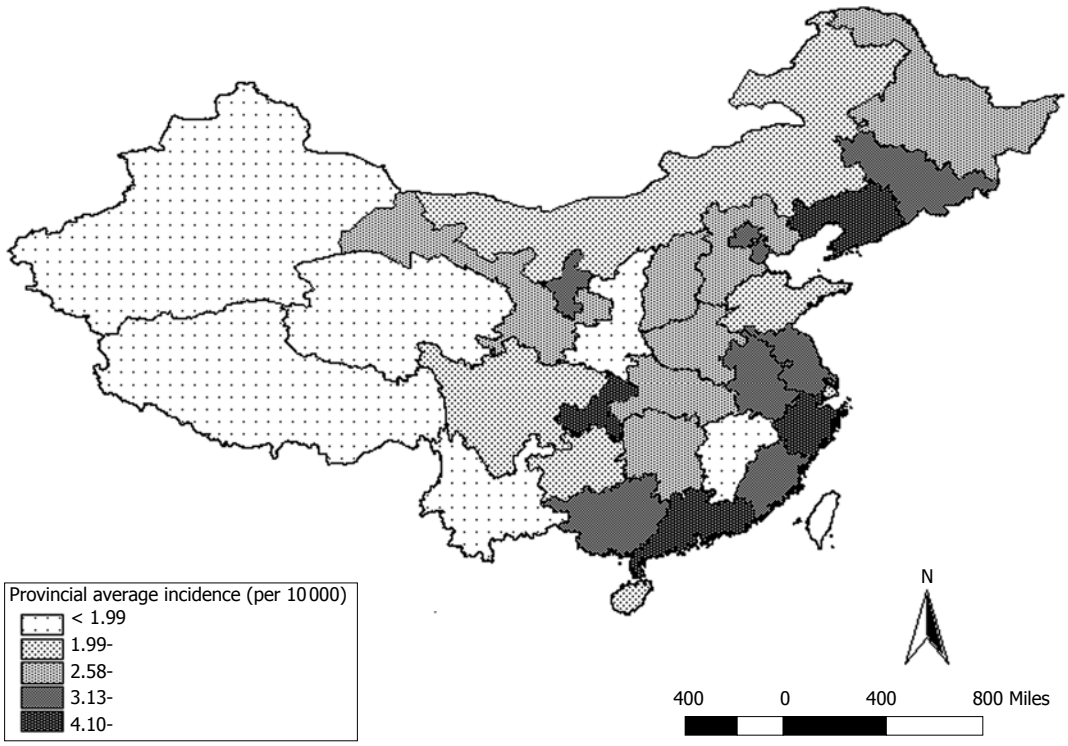

Figure 1 National distribution graph of average incidences of anorectal atresia/stenosis in different provinces, municipal cities and autonomous regions from 2001 to 2005.

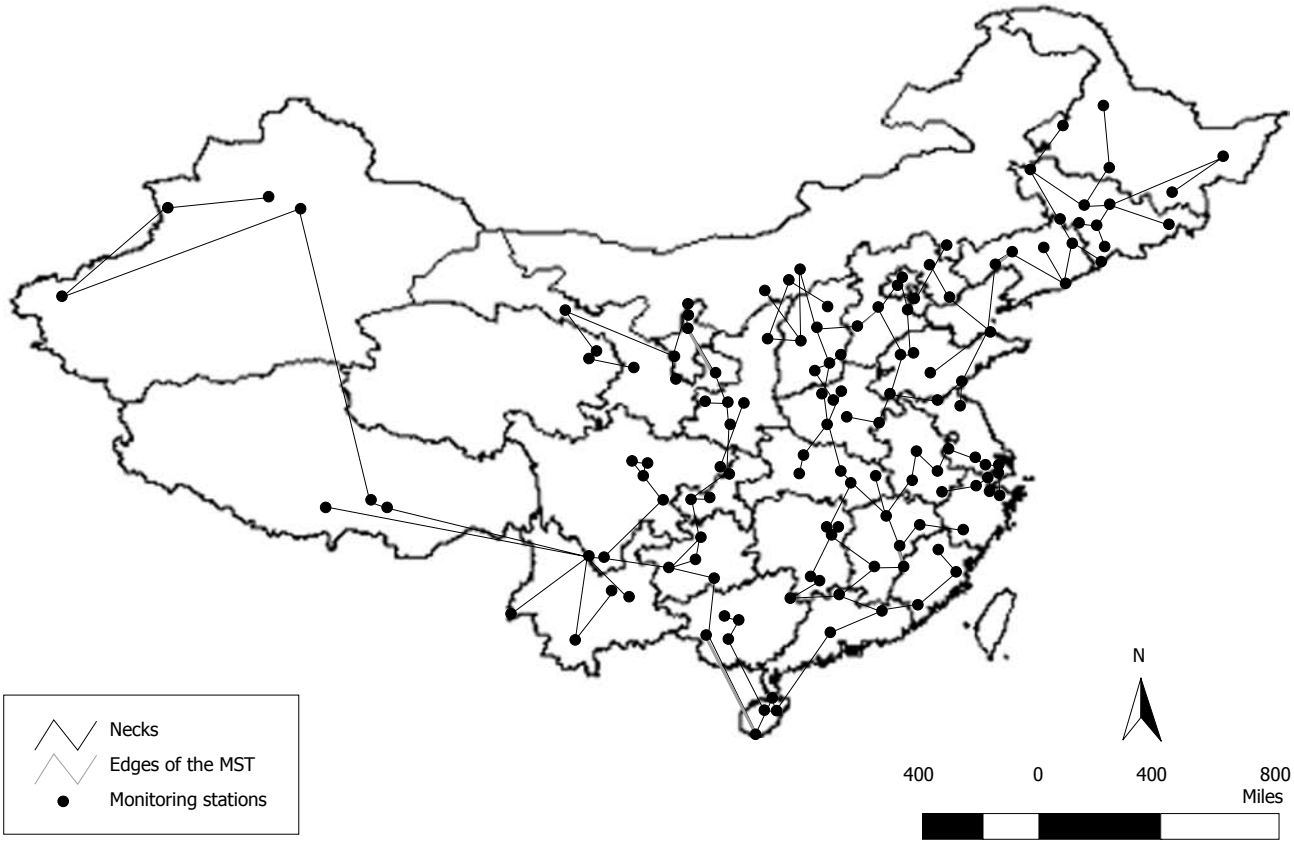

Figure 2 Two-dimensional MST-based cluster graph of monitoring sites in China from 2001 to 2005.

and south Jiangxi Province; Cluster 3 consisted of those in Beijing Municipal City, Tianjin Municipal City, Hebei Province, Shandong Province, north Jiangsu Province, and north Anhui Province; Cluster 4 was made up of those in Zhejiang Province, Shanghai Municipal City, south Anhui Province, south Jiangsu Province,

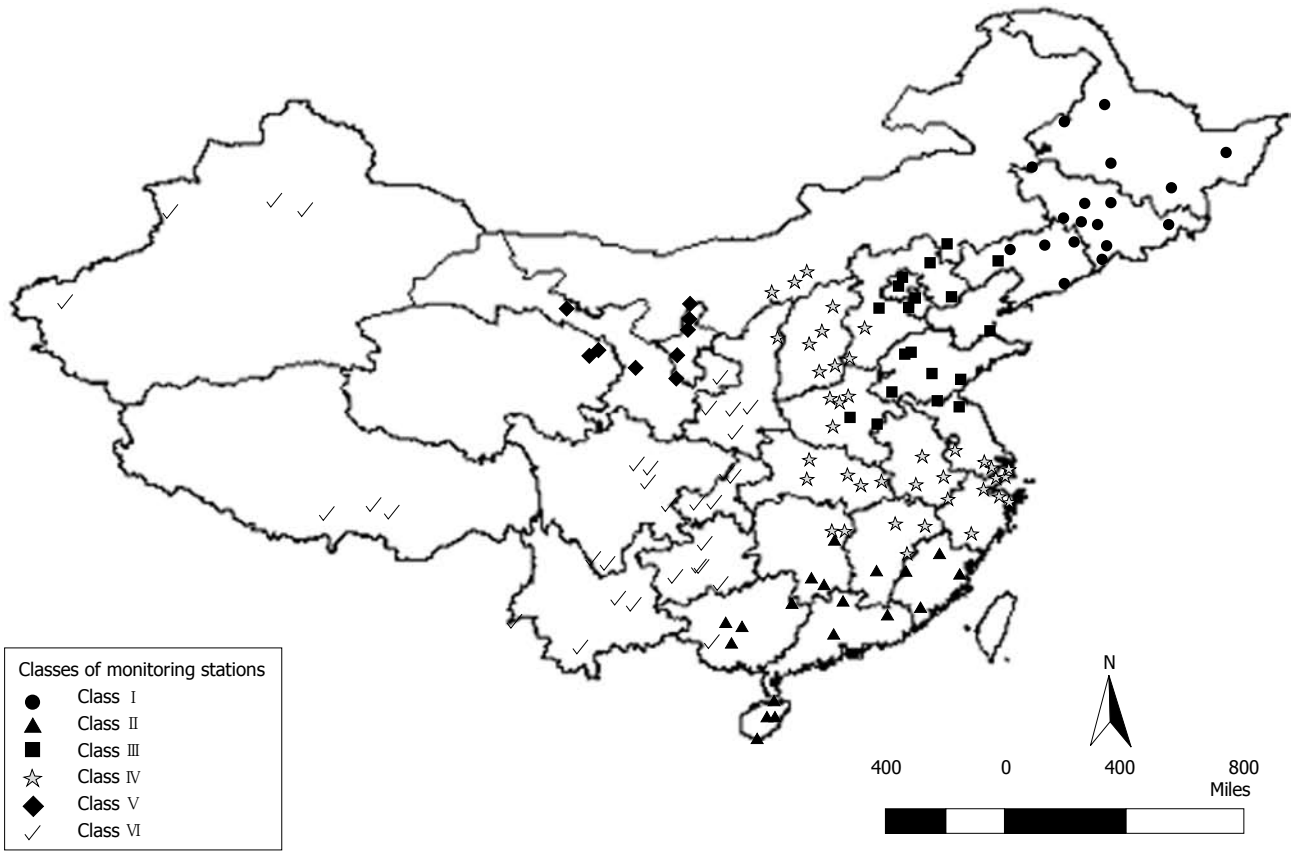

Figure 3 Cluster graph of monitoring sites in China from 2001 to 2005.

north Hunan Province, north Jiangxi Province, Hubei Province, Henan Province, Shanxi Province and Inner Mongolia Autonomous Region; Cluster 5 consisted of those in Ningxia Hui Autonomous Region, Gansu Province and Qinghai Province; and Cluster 6 included those in Shaanxi Province, Sichuan Province, Chongqing Municipal City, Yunnan Province, Guizhou Province, Xinjiang Uygur Autonomous Province and Tibet Autonomous Region (Figure 3).

DISCUSSION

Anorectal atresia/stenosis is one of the most common malformations in the gastrointestinal tract. Due to pathological changes in the anus and rectum, one-third of the perinatal births with anorectal atresia/stenosis suffer from defecation difficulties of varying degrees of severity following surgery. Most of these births need life-long treatment that severely compromises the quality of life and psychological development in particular. This situation is a burden not only to these babies, but also to their entire families and even to society as a whole in China[13-17]. Some researchers[18-22] suggested that mothers' contact (when they are pregnant) with environmental pollutants could increase their risk of giving birth to babies having congenital malformations. The current research found that the areas with the highest incidences of anorectal atresia/stenosis were concentrated in Eastern China, especially in Liaoning, Zhejiang and Guangdong. With a solid industrial and agricultural base, economic conditions in Eastern China flourish. Most manufacturing plants and industrial factories (including marine-aquatic industries) are located in Eastern China. It is known that these factories are responsible for water pollution and other industrial pollution at a level that is deemed severe. Perhaps mothers in Eastern China have babies with more congenital malformations because of the mothers' severe exposure to these physical and chemical pollutants when they are pregnant. In addition, the regional differences in awareness and uptake of available health care for pregnant woman, infrastructure of monitoring hospitals and diagnosis at a technical level were also factors likely to explain some of the observed geographical variation in anorectal atresia/stenosis. In Western China, limited at economic and cultural levels, most pregnant woman have weak awareness and uptake of health care, which means they do not actively seek antenatal care, so there is the probability of under-reporting of cases, resulting in the lower incidence. As to the health services, in the less developed western regions, the maternal and child healthcare facilities may lack necessary infrastructure, and the technical levels of monitoring staff may be limited, which may also result in the lower detection of congenital malformation.

Cluster analysis is an exploratory data analysis tool for solving classification problems. Assuming the samples as the vectorial points in hyperspace, the object of cluster analysis is to sort the samples into clusters so that the degree of association is strong between members of the same cluster and weak between members of different

clusters. It has widespread application because of its advantage of definite classification. In analysis of spatial distribution structures of disease, both the similarities and the adjacent relationships of geographic units of the same cluster are of interest to researchers[23]. The traditional cluster analysis cannot meet all the requirements. Nevertheless, the two-dimensional graph-theoretical clustering model systematically (1) combines the concept of the two-dimensional constrained spatial hierarchical clustering and the MST method in the graph theory; (2) utilizes the spatial analysis measures of Geographic Information System (GIS) in combination with the tree algorithm to divide the geographic units into clusters. This model allows researchers to consider the similarities as well as the spatial connectivity between different units in the same cluster. This is of significance in (1) analyzing the similarities of different geographic units, (2) demonstrating the spatial distribution of the disease, and (3) identifying the boundaries of the spatial heterogeneity of the disease. Luo *et al*[24] combined the principal component analysis and two-dimensional graph-theoretical clustering to identify the evaluation method for land consolidation priority. Cao *et al*[25] divided the national corn reserve regions based on the two-dimensional graph-theoretical clustering, providing references for application of regional corn reserve technology and for the formation of guidelines for macro-regional corn reserve technology. Few researchers, however, have reported on the use of two-dimensional graph-theoretical clustering as applied to study the spatial distribution of congenital malformations.

This research utilized the two-dimensional graph-theoretical clustering to divide monitoring sites of the CBDMN into different clusters of areas based on average incidences of anorectal atresia/stenosis. The findings in this research will have important guiding significance for further analysis of relevant environmental factors regarding anorectal atresia/stenosis and for allowing regional monitoring for anorectal atresia/stenosis. On the one hand, the congenital malformations relate not only to genetic factors, but also correspond with the influence of other conditions: geographic environment, climate, economic development and even cultural development[26-30]. The results from the two-dimensional graph-theoretical clustering will enable epidemiologists to determine which environmental factors affect the incidence of anorectal atresia/stenosis in each cluster of areas by considering their respective environmental characteristics. On the other hand, although these data showed high incidence of anorectal atresia/stenosis in Eastern China and low incidence in Western China, it is true that different areas within Eastern China and Western China have their own demographic, economic and environmental characteristics. Large-scale monitoring cannot obtain detailed influential factors of anorectal atresia/stenosis in any given region. The results in this research provide an approach for researchers to monitor relevant environmental influential factors for incidence of anorectal atresia/stenosis regionally. By dividing the monitoring sites of the CBDMN into different clusters, the detailed relevant environmental risk factors for anorectal atresia/stenosis in different geographic units can be collected within the same cluster to allow regional monitoring.

The current research took account of the adjacent relationship between different monitoring sites rather than different provinces, autonomous regions or municipal cities, which guaranteed the requirements for geographic divisions for this study. However, if different monitoring sites in the same province were incorporated into different clusters after two-dimensional graph-theoretical clustering, the monitoring work at the provincial level would be subjected to increased difficulties.

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COMMENTS

Background

The incidence of anorectal atresia/stenosis is high amongst gastrointestinal tract malformations. It relates not only to genetic factors but also to environmental factors, especially spatial differences. However, very little information available in literature about the spatial distribution patterns of anorectal atresia/stenosis in China.

Innovations and breakthroughs

This is the first study to report the spatial distribution of anorectal atresia/stenosis in China using two-dimensional graph-theoretical clustering considering simultaneously the similarities as well as the spatial connectivity between different units in the same cluster.

Applications

The findings will have important guiding significance for further analysis of relevant environmental factors of anorectal atresia/stenosis and for allowing regional monitoring for anorectal atresia/stenosis.

Terminology

Anorectal atresia/stenosis: a congenital malformation characterized by absence of continuity of the anorectal canal or of communication between rectum and anus, or narrowing of anal canal, with or without fistula to neighboring organs. Two-dimensional graph-theoretical clustering: a cluster method of combining the concept of the two-dimensional constrained spatial hierarchical clustering and the MST method in the graph theory, and utilizing the spatial analysis measures of geographic information system (GIS) in combination with the tree algorithm to divide the geographic units into clusters.

Peer review

The study is based on 460 hospitals from 138 cities across China, and is an interesting paper on the geographical distribution of anorectal atresia/stenosis across China.

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BRIEF ARTICLES

Effect of *p27mt* gene on apoptosis of the colorectal cancer cell line Lovo

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Author contributions: Chen J designed the research; Chen J, Wang JN, Ding WH, Huang YZ performed the research and analyzed the data; Chen J wrote the paper; Xu SY and Deng CS supervised the whole study.
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Abstract

AIM: To construct *p27mt* recombinant adenovirus, transfect the colorectal cell line Lovo and observe the effects of *p27mt* on Lovo cell apoptosis and cell cycle inhibition.

METHODS: We constructed recombinant adenovirus containing *p27mt* by homologous recombination in bacteria. The colorectal cancer cell line Lovo was infected with recombinant replication-defective adenovirus Ad-p27mt, and expression of *p27mt* was determined by Western blotting; the inhibitory effect of *p27mt* on Lovo cells was detected by cytometry. Cell cycle was determined by flow cytometry. DNA fragment analysis identified the occurrence of apoptosis.

RESULTS: The recombinant adenovirus which already contained *p27mt* target gene was successfully constructed. When multiplicity of infection was $\geqslant$ 50, the infection efficiency was 100%. After transfection of Lovo cells with Ad-p27mt the cells had high *p27* expression which was identified by immunoblotting assay. PI staining and flow cytometry showed that 77.96% of colorectal cancer cells were inhibited in phase G_0/G_1, while in the Ad-LacZ group and blank control group, 27.57% and 25.29% cells were inhibited in the same phase, respectively. DNA fragment analysis, flow cytometry and TUNEL assay demonstrated that *p27mt* is able to induce apoptosis in colorectal cancer cells.

CONCLUSION: *p27mt* has an obvious blocking effect on colorectal cancer cell cycle, and most cells were inhibited in phase G_0/G_1. Therefore, *p27mt* can induce apoptosis in colorectal cells.

Key words: Apoptosis; Cell cycle; Colorectal cancer; *p27mt*; Recombinant adenovirus

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INTRODUCTION

p27Kip1 (*p27*) is a cyclin dependent kinase inhibitor (CDKI), whose specific late G_1 destruction allows progression of the cell across the G_1/S boundary. The protein was ubiquitinated by S-phase kinase-interacting protein-2 (Skp2) following its specific phosphorylation, and was subsequently degraded by the 26S proteasome[1]. There was a direct relationship between the low level of *p27* and rapid proliferation occurring in several benign states and in many malignancies. It has been reported that *p27* levels were markedly reduced in several malignancies, such as those of the skin[2], liver[3], bladder[4], thyroid[5], breast[6], prostate[7] and endometrium[8]. In some of the tumors studied, a strong correlation was found between the low level of *p27*, the aggressiveness of the disease and poor prognosis of the patients[6]. Interestingly, *p27* in all these tumors was of the wild-type species (*p27wt*), and its regulation has been attributed to phosphorylation of Thr-187 and subsequent

ubiquitination[9]. Overexpression of *p27 via* adenoviral gene transfer could suppress cancer cell growth regardless of *p27* mutation[10]. Montagnoli *et al*[11] showed that the ubiquitination of *p27* did not occur in *p27mt* with Thr-187 to Ala [p27 (T187A)]. Sheaff *et al*[12] showed that the transfection of p27 (T187A) plasmid caused a G_1 block, which was both resistant to and not modulated by cyclin E/Cdk2. On the basis of these observations of *p27* regulation and the nature of the *p27* tumor suppressor gene, we constructed Adenovirus expressing *p27mt* (Thr-187/Pro-188 to Met-187/Ile-188) to infect the colorectal cancer cell line Lovo, and then investigated its expression and functional significance in the cell proliferation and apoptosis of Lovo cells, by which we aimed to discuss novel methods of gene therapy in colorectal cancer.

MATERIALS AND METHODS

Main reagents

The restriction endonucleases such as *Age* I , *Nhe* I , *Kpn* I , *Pac* I and *Pme* I were purchased from New England Biolabs Co. *Hin*dⅢ, *Eco*RI, λDNA *Hin*dⅢ marker, 200 bp DNA ladder, dNTP, Tag enzyme and T4 DNA ligase were purchased from Huamei Biological Co. (China). The Western blotting kit was purchased from KPL Co. (USA). The rat anti-human *p27kip1* multi-antibody was purchased from Santa Cruz Co. (USA). The horseradish peroxidase (HRP) labeled sheep anti-rat IgG monoclonal antibody was purchase from Zhongshan Co. (China). The *p27mt* primer was designed and synthesized by Beijing Saibaisheng Biological Co. (China). The fetal bovine serum (FBS) was purchased from Hangzhou Sijiqing Biological Engineering Materials Co. (China). The liposome (polyfect) was purchased from Qiagen Co. (USA). Trypsin, DMEM culture medium, Hepes and Cscl were purchased from Sigma Co. (USA).

Plasmid, strain, adenovirus and cell lines

The pORF9-p27mt plasmid was purchased from Invivogen (USA). The pAdeasy-1 plasmid, pBluescript Ⅱsk (+), Ad293 cell, *E. coli* BJ5183 and XL10-gold were purchased from Stratagene (USA). The LacZ recombinant adenovirus (Ad-LacZ with titer 7.15×10^{15}/L)[13] and DH5α were gratefully provided by Doctor Wang Jianing, Clinical Research Institute, Yunyang Medical College. The Lovo cell line was purchased from Type Culture Collection Center, Wuhan University.

Main equipment

This equipment included a high speed freezing centrifuge (Universal 32R, Germany), the ultraspeed freezing centrifuge (Tokyo Cp80max, Japan), inverted phase contrast microscope (Nikon TE2000-u, Japan), CO_2 culture box (CB150#00-17611, wtb-binder), PCR machine (Biometra. Germany), ultraviolet spectrophotometer (Auriud CE2401, UK), Coulter Epics XL flow cytometer (Beckman Co., USA), high speed table-top centrifuge and a water bath shaking table (China).

Construction and identification of p27mt recombinant adenovirus

After pORF9-p27mt was digested by *Age* I and *Nhe* I enzymes, the 619 bp fragment was recycled and subcloned into pBluescript ⅡSK (+) which was digested by *Xma* I and *Xba* I enzymes, thus obtaining pBluescript-p27mt. Then pBluescript-p27mt was digested by *Not* I and *Kpn* I enzymes, and the 699 bp fragment was recycled and inserted into the shuttle plasmid vector pShuttle-CMV which was digested by the same enzymes, thus the transfer plasmid pShuttle-CMV-p27mt was obtained. The competent *E. coli* was transformed by the adenoviral framework plasmid pAdeasy-1. According to the ampicillin-resistant gene, the BJ5183 containing pAdeasy-1 was picked out and prepared into the ultra-competence BJ5183 containing pAdeasy-1. Then, the ultra-competence BJ5183 was transformed by transfer plasmid pShuttle-CMV-p27mt which was digested by *Pme* I enzyme and dephosphorylated by alkaline phosphatase. A little DNA from the transformed clone bacterial plasmid was taken out and the suspect DNA of the recombinant adenovirus plasmid was chosen according to the size of the plasmid in agarose electrophoresis. If the chosen DNA was identified as the correct DNA by digestion of *Pac* I enzyme, then the recombinant adenovirus plasmid pAdeasy-1-p27mt could be prepared. Recombinant adenovirus plasmid DNA was excised by *Pac* I , then transfected by Ad293 *via* liposome polyfect mediation, where the change in cells at different times after transfection was determined. When it appeared that 90% had cell lesions, scratch 293 cells from the culture bottle were vortexed three times at -80℃ to +37℃ to lyse the cells, then centrifuged and the supernatant containing the virus was collected, the 293 cells were reinfected with the above virus and proliferation of the virus occurred at a large scale. Purification of recombinant adenovirus was similar to the method of Cortin *et al*[14]. After purification the adenovirus underwent dialysis, test virus titers were detected using an ultraviolet spectrophotometer. Fifty microliter of purified adenovirus liquid, 100 g/L SDS 20 μL, PBS 430 μL, were assayed at absorbance values of A_{260} and A_{280}, then the granule numbers and purity of adenovirus were determined. If $A_{260} = 10^{15}$pfu/L and $A_{260}/A_{280} > 1.3$ this indicated that the purity was relatively high when virus titers (pfu/L) = $A_{260} \times$ dilution $\times 10^{15}$. PCR identification of recombinant adenovirus Ad-p27mt was carried out. Recombinant adenovirus genome DNA from the high titer virus storage liquid served as a template. Using primer toward the reporter gene *p27mt*, the PCR reaction parameters were: pre-denaturation at 95℃ for 5 min, denaturation at 94℃ for 20 s, annealing at 56℃ for 30 s, elongate at 72℃ for 30 s, 30 cycles, elongate at 72℃ for 10 min. Primer 1: 5'CCTAGAGGGCAAGTACGAGTG3'; Primer 2: 5'GAAGAATCGTCGGTTGCAGGTCGCT3'.

Detection of p27mt gene expression

Lovo cells were incubated in 100 mL/L FCS and RPMI-1640 culture medium at 37℃ in a 50 mL/L CO_2 culture box until the cells spread to 70%-80% of the area and were used in the following experiment.

Lovo cells taken from the 15 cm culture flask were infected with Ad-LacZ according to an Multiplicity of infection (MOI) of 20, 40, 50 and 100 and then incubated for another 48 h. The cells were then fixed by 5 mL/L glutaral for 15 min and washed three times with PBS,

X-gal staining solution (20:1) was added. The cells were then incubated at 37℃ in the 50 mL/L CO_2 culture box for 4-24 h. The blue-stained cells, i.e. the positive cells in which the LacZ gene was expressed, were observed under the microscope and the percentage of positive cells was calculated.

Lovo cells taken from the 75 cm culture flask were infected with Ad-p27mt (MOI 50) and Ad-LacZ (MOI 50), respectively. After incubation in the same conditions for 48 h, the cells were digested by 0.5 g/L trypsin, collected and washed twice with PBS. After lysis by 500 μL 1 × SDS-PAGE cell lysis solution and boiling for 5 min, the cells were centrifuged, the supernatant was collected and then detected by Western blotting.

Cycle detection and apoptosis of cells infected with Ad-p27mt by flow cytometry

Lovo cells cultured in the 75 cm culture flask were infected with Ad-p27mt (MOI 50). After incubation for 48 h, the cells were digested by 0.5 g/L trypsin, collected and then washed twice with PBS. The cell concentration was adjusted to 10^9/L with PBS. One hundred microliter of cell suspension was taken out and mixed with 200 μL DNA-PREP™ LPR and placed at room temperature in the dark for 3 min. The cell suspension was then mixed with 1000 μL DNA-PREP stain (PI staining). The cell cycle phase and apoptosis were detected by a Coulter Epics XL flow cytometer 15 min later. Ad-LacZ (MOI 50) group and normal controls (Lovo cells cultivated without adenovirus) were used as control groups.

Apoptosis by DNA fragment analysis

The cells in the three groups (Ad-p27mt and Ad-LacZ for 48 h and the normal control), were collected and centrifuged at 1000 r/min for 5 min. The supernatant was discarded, and 500 μL of cell lysis solution [1% Np40, 20 mmol/L EDTA, 50 mmol/L Tris-HCl (pH7.5)] and 10 μL protease K were added to the cell sediment. Following incubation in a water bath (56℃) for 1-2 h and extraction with phenol and chloroform, DNA was precipitated by dehydrated alcohol. After washing once with 700 mL/L alcohol, 200 μL TE was added to lyse the DNA. Then RNase (final concentration 50 μg/mL) was added and placed at 37℃ for one night. The DNA was electrophoresed in 10 g/L agarose gel and the results were observed under an ultraviolet lamp.

Detection of cell apoptosis by the TUNEL method

1×10^4 cell suspension was inoculated into a 60 mm dish with a cover glass (washed and high-pressure sterilized). Each of the Ad-p27mt group and the normal control were inoculated into 6 glasses and incubated for 24 h. The glasses were then taken out and washed twice with 1 × PBS and fixed with methanol and freezing acetic acid (3:1) for 30 min. The next procedure was carried out according to the kit instructions. One thousand cells were counted on each glass and the average number of apoptotic cells was determined. Then the apoptotic index (AI), i.e. the number of apoptotic cells in every 100 cancer cells, was calculated.

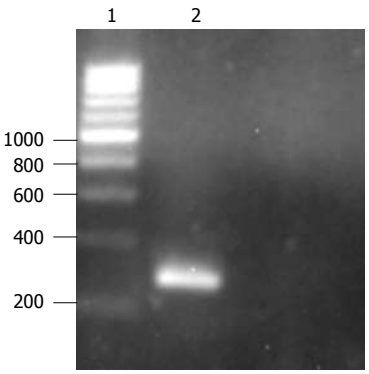

Figure 1 Identification of Ad-p27mt by PCR. 1: 200bp marker; 2: Ad-p27mt.

Statistical analysis

One way-ANOVA was used in processing the measured data, which were expressed as mean ± SD. χ^2 test was adopted in the calculation of enumeration data.

RESULTS

PCR identification of the recombinant adenovirus Ad-p27mt

The pathologically changed 293 cells and the culture fluid were collected, frozen and thawed three times and centrifuged. Five millilitre of the supernatant was taken out and added to 1 mg protease K, 2 mL 1% SDS, 10 mmol/L EDTA and 20 mmol/L Tris-HCl for 2 h. After centrifugation, the supernatant was extracted with phenol and chloroform. After precipitation with dehydrated alcohol, the viral DNA was collected. Then forward and reverse primers were added and the PCR reaction was carried out. The 275 bp target gene was amplified, which showed that the recombinant adenovirus already contained the *p27mt* target gene (Figure 1).

X-gal chemical staining

After Lovo cells were infected with Ad-LacZ, the adenovirus-mediated gene transfer rate was evaluated by X-gal staining. The results showed that the infection efficiency could reach 100% when MOI was larger than 50, which indicated that recombinant adenovirus could effectively transfer the gene to Lovo cells *in vitro* (Figure 2).

The expression of p27 protein was evaluated after Lovo cells were infected with human mutant p27 recombinant adenovirus in vitro

After Lovo cells were infected with Ad-p27mt (MOI 50) for 24 h, these cells were collected and lysed using 1 × SDS × PAGE cell lysis solution. After boiling at 100℃ for 5 min, the cells were centrifuged. The supernatant was collected and the protein was detected by the TMB system Western blotting kit (KPL, USA). After staining with TMB stain, a high expression of 27 KD protein was observed in the Ad-p27mt group while only slight expression (endogenous expression) was observed in the Ad-LacZ group and the normal control group. This showed that the *p27mt* recombinant adenovirus constructed in the present study could express *p27mt* gene in Lovo cells and the protein could also be expressed at a high level in Lovo cells (Figure 3).

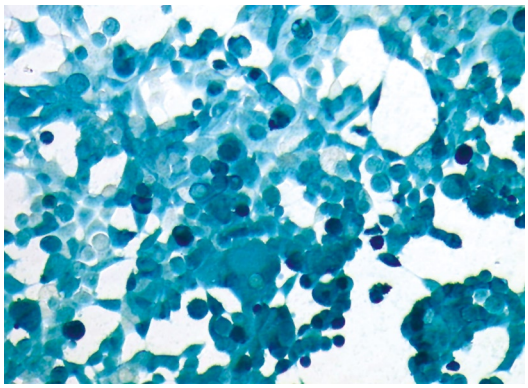

Figure 2 X-gal chemical staining detected the infection efficiency of Ad-p27mt (MOI 50).

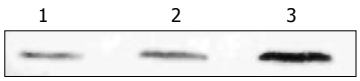

Figure 3 Western blotting of expressed p27 protein. 1: Non-infected Lovo cells; 2: Ad-lacZ infected Lovo cells; 3: Ad-p27mt infected Lovo cells.

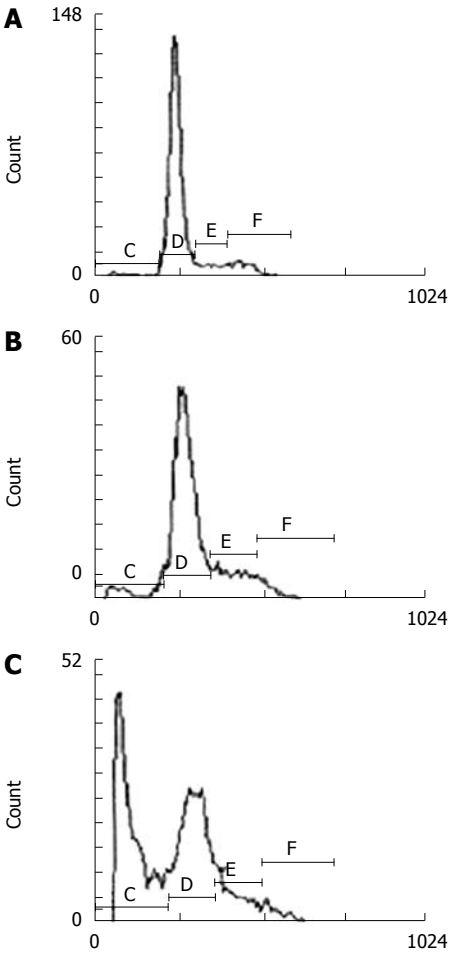

Figure 4 Determination of cell apoptosis by Flow cytometry. A: Non-infected Lovo cells; B: Ad-lacZ infected Lovo cells; C: Ad-p27mt infected Lovo cells.

Apoptosis of colorectal cancer cells induced by Ad-p27mt

After Lovo cells were treated with Ad-p27mt, Ad-LacZ and without virus for 24 h, apoptosis was observed by flow cytometry and was repeated six times. The average value of hypodiploid cells was: 41.0%, 4.67% and 1.96%, respectively. After statistical analysis, there was a significant difference among the three groups, ($P < 0.01$) (Figure 4).

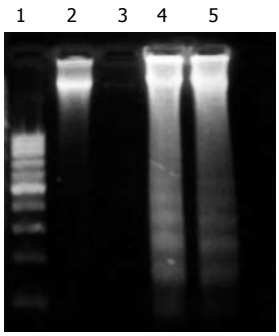

Figure 5 Determination of cell apoptosis by DNA fragment analysis. 1: Marker; 2: Blank group; 3: Ad-lacZ group; 4, 5: Ad-p27mt group.

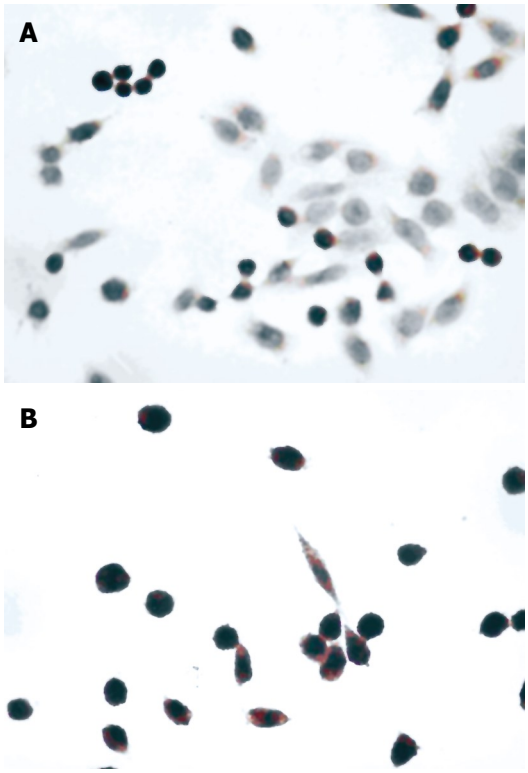

Figure 6 Detection of cell apoptosis by the TUNEL method. A: Blank group; B: Ad-p27mt.

Detection of DNA fragments

The results of DNA electrophoresis showed that the gene bands were intact in the Ad-LacZ and normal control group, while there were obvious 180-200 bp diploid "trapezia" bands in the Ad-p27mt infected group, which was in concordance with the characteristic changes of apoptosis (Figure 5).

Detection of cell apoptosis by the TUNEL method

The nuclei of apoptotic cells were dark stained, the cytoplasm was concentrated and the cells had shrunk. The AI of the Ad-p27mt and the control group were (82.6% ± 3.2%) and (5.0% ± 3.5%), respectively and showed a significant difference ($P < 0.05$). This demonstrated that Ad-p27mt could obviously induce apoptosis in colorectal cancer cells (Figure 6).

Table 1 The effect of Ad-p27mt on the cell cycle of Lovo cells (mean ± SD)

Group	G_0/G_1	S	G_2/M
Blank group	25.29 ± 1.04	41.12 ± 1.19	33.34 ± 1.55
Ad-LacZ group	27.57 ± 0.45[b]	38.21 ± 0.44[b]	34.22 ± 0.92[b]
Ad-p27mt group	77.96 ± 2.20[d]	8.98 ± 0.17[d]	13.06 ± 2.35[d]

Hypodiploid cells were not included ([b]$P < 0.01$ *vs* Ad-LacZ group, [d]$P < 0.01$ *vs* Blank group).

The effect of exogenous p27mt on the cell cycle (Table 1)

The cell cycle of Lovo cells after the various treatments are shown in Table 1. It can be seen that in the Ad-LacZ and blank control groups, the number of cells in the G_0/G_1 phase decreased gradually and the percentage of cells in the S phase increased, which indicated that the transition time of the cell cycle was shortened and cell proliferation was active. However, the percentage of cells in the G_0/G_1 phase decreased and the percentage of cells in the S phase increased and the cell cycle was arrested in the G_0/G_1 phase in the Ad-p27mt group, which was significantly different from the blank and Ad-LacZ groups ($P < 0.01$).

DISCUSSION

Currently, functional reconstruction of anti-oncogenes is a reasonable strategy for tumor gene therapy. p27 protein degradation is mainly caused by phosphorylation of the 187th threonine of *p27* which is mediated by ubiquitin[15-17]. Park *et al*[18,19] replaced the 187th threonine and the 188th praline of the wild-type *p27* with methionine and isoleucine, respectively, by which the target phosphorylation site of CDK would be protected from phosphorylation, and thus the mutant *p27* was prepared. These authors constructed a replication-deficient recombinant adenovirus which carried *p27mt* and *p27wt*, respectively and proved that the inhibition and apoptotic effects in cancer cells was more obvious in the mutant *p27 (p27mt)* than in the wild-type *p27 (p27wt)*.

This study constructed a replication-deficient recombinant adenovirus which successfully carried *p27mt* (Ad-p27mt). Ad-p27mt was then transfected into Lovo cells. When the MOI ⩾ 50, the infection efficiency was 100%. Using Western blotting, high expression of *p27* was observed, and using FACS, the rate of apoptosis was up to 41.0% in the Ad-p27mt group which was significantly different compared to the control group ($P < 0.01$). DNA analysis showed a 180-200 bp DNA ladder, and using the TUNEL technique, the apoptotic index was sharply upregulated to 82.6% which was significantly different compared to the control group ($P < 0.05$). These results showed that *p27mt* is an important gene and is related to the incidence of colorectal carcinoma. The downregulation of *p27* may be the main cause of cell differentiation dysfunction and apoptosis dysfunction. Mutant *p27* promoted the expression of *p27* in Lovo cells, which led to apoptosis in tumor cells. This may be a potential new therapy for colorectal carcinoma.

The concentration of cyclin/CDK is the key factor for cells passing the G_1-S threshold. Cell cycle analysis showed that the cleavage of tumor cells was stopped at the G_1 stage by *p27mt* suppressing the activity of the cyclin/CDK kinase. Hurteau *et al*[20] reported that the accumulation of *p27* played a role in the cell cycle arrest mechanism at the initiation of cell differentiation. A related report[21] in China showed that the *p27* gene suppressed DNA replication and protein synthesis and reduced cell mitosis division and inhibited cell generation.

In our study, apoptosis of colorectal carcinoma cells was successfully induced by the application of the mutant gene p27. The apoptosis rate was significantly higher than that of wild-type p27 reported in our previous study[21], which serves as a very useful experimental support for tumor suppression function reconstruction in the gene therapy of colorectal carcinoma. The efficacy of this method *in vivo* and the mechanism of apoptosis should be determined in future studies.

ACKNOWLEDGMENTS

We thank Dr. Xiao-Jun Yang for his excellent technical assistance.

COMMENTS

Background

Along with the improvement in living standards and a change in diet, there has been a gradual increase in the incidence of colorectal cancer in China. However, no effective therapeutic modalities are available for this condition. Gene therapy for the restoration of *p27* expression is a promising therapy. A mutant type *p27* gene, with a mutation of Thr-187/Pro-188 to Met-187/Ile-188, can inhibit degradation of p27 protein by the ubiquitin-mediated pathway. Inhibition of mutant *p27* (*p27mt*) in tumor cells was stronger than that in wild-type *p27* (*p27wt*), which was demonstrated by cells arresting in the G_0/G_1 phase. In addition, the apoptosis promoting activity of *p27mt* was also stronger. However, few studies on the *p27* gene in colorectal cancer have been reported.

Research frontiers

p27, as a cyclin-dependent kinase inhibitor, tumor suppressor gene, and promoter of apoptosis, has been widely investigated in malignancies such as skin, liver, bladder, thyroid, breast, prostate and endometrium cancer. However, the apoptotic bioactivity of *p27mt* has not been studied in colorectal cancer.

Innovations and breakthroughs

The study indicates that Ad-*p27mt* has a strong apoptosis inducing bioactivity as well as a cell cycle inhibitory effect in colorectal cancer *in vitro*.

Peer review

This is a nice article. This *in vitro* effect of *p27mt* should be examined *in vivo* to determine the safety and efficacy.

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BRIEF ARTICLES

Expression of semaphorin 5A and its receptor plexin B3 contributes to invasion and metastasis of gastric carcinoma

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Abstract

AIM: To investigate the protein and mRNA expression of semaphorin 5A and its receptor plexin B3 in gastric carcinoma and explore its role in the invasion and metastasis of gastric carcinoma.

METHODS: Expression of semaphorin 5A and its receptor plexin B3 in 48 samples of primary gastric carcinoma, its corresponding non-neoplastic mucosa, and matched regional lymph node metastasis was assayed by reverse transcription-polymerase chain reaction (RT-PCR), real-time RT-PCR and Western blotting.

RESULTS: The protein and mRNA expression of semaphorin 5A and its receptor plexin B3 increased gradually in non-neoplastic mucosa, primary gastric carcinoma and lymph node metastasis ($P < 0.05$). Moreover, the expression of semaphorin 5A was closely correlated with that of plexin B3.

CONCLUSION: Semaphorin 5A and its receptor plexin B3 play an important role in the invasion and metastasis of gastric carcinoma.

Key words: Semaphorin 5A; Plexin B3; Gastric carcinoma; Invasion; Metastasis

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INTRODUCTION

Gastric cancer is the leading cause of cancer-related death in some Asian countries including China and Japan. Despite the advances in treatment and research efforts over the past few decades, the outcome of gastric cancer remains poor. The overall 5-year survival rate of gastric cancer patients is 5%-15% in the United States and most other Western countries, largely because many gastric cancers are diagnosed at an advanced stage. The aggressive nature of human gastric carcinoma is related to a variety of intracellular events, including activation of various oncogenes, inactivation of tumor suppressor genes. Therefore, great attention has been given to endogenous factors of tumors, which appear to be responsible for tumor cell growth, progression, and invasion. Identification of such endogenous factors not only leads to a better understanding of the carcinogenesis and development of gastric cancer, but also provides new strategies for developing agents that specifically suppress this process.

Semaphorins are the products of a large family of genes currently containing more than 30 members, all of which share a conserved N-terminal domain called the "sema" domain, a segment of approximately 400-500 amino acids[1]. Based on sequence similarity and distinctive structural features, these genes have been grouped into eight subclasses[1-8], of which classes 3-7 are the products of vertebrate semaphorins. Plexins

are identified as the best characterized semaphorin receptors, which are segregated into four sub-families containing nine members. It has been shown that some vertebrate semaphorins belonging to classes 4-7 can bind directly to plexins and activate plexin-mediated signal transduction[2,3]. These semaphorins and plexins have been originally characterized as constituents of the complex regulatory system responsible for the guidance of axons during the development of the central nervous system[4,5]. However, a growing body of evidence suggests that certain semaphorins, through interacting with its receptors, play a regulatory role in the occurrence and development of tumor[6-9]. Semaphorin 5A is a member of class 5 semaphorins. Plexin B3, belonging to class B plexin subfamily, is a receptor for semaphorin 5A[10]. However, it is unclear whether semaphorin 5A exerts certain biological functions in the progression of human cancers including gastric carcinoma through plexin B3.

In the present study, we investigated the protein and mRNA expression of semaphorin 5A, plexin B3 in primary gastric carcinoma as well as in its corresponding non-neoplastic mucosa and matched regional lymph node metastasis, and preliminarily analyzed their relation with the invasion and metastasis of gastric cancer.

MATERIALS AND METHODS

Patients and specimens

Forty-eight advanced gastric adenocarcinoma (TNM stage Ⅲ-Ⅳ) patients (28 male and 20 female) with lymph node metastasis diagnosed by postoperative pathology were investigated in this study. Their mean age was 58.7 years (range 45-68 years). The patients received neither chemotherapy nor radiation therapy prior to tumor resection and provided their consent for use of tumor tissue. Tissue blocks of non-neoplastic mucosa (> 5 cm away from the edge of tumor), primary tumor and its corresponding metastatic lymph nodes were obtained within 30 min after they were removed from the patients. Each block was cut into two pieces, one for routine pathologic diagnosis and the other for molecular analysis. Samples were frozen in liquid nitrogen immediately and stored at -260℃ until use.

Reverse transcription-polymerase chain reaction (RT-PCR)

Tissues were lysed using Trizol reagent (Invitrogen, Carlsbad, CA), and total RNA was isolated using chloroform and isopropyl alcohol according to the manufacturer's instructions. After RNA was quantified, 1-5 μg of RNA was annealed to Oligo (dT) at 65℃ for 5 min and cooled at room temperature. Using a proSTAR first strand RT-PCR kit (Stratagene, La Jolla, CA, USA), reverse transcriptase and dNTPs were added to the RNA-Oligo (dT) mixture and the reaction was performed at 42℃ for 1 h. Each single-strand cDNA was used for subsequent PCR amplification of semaphorin 5A, plexin B3 and β-actin with the latter used as a quantitative control. PCR was carried out in a reaction volume of 25 μL under the following conditions: an initial denaturation at 95℃ for 5 min, followed by 30 cycles at 94℃ for 30 s, at 55℃ for 50 s, at 72℃ for 40 s, and a final extension at 72℃ for 5 min on an authorized thermal cycler. The primer sequences used amplification are 5'-CTCAGTCGCTGTGAGCG TTAT-3' and 5'-CAGATGTTGGACCGCCAAATA-3' for semaphorin 5A, 5'-TCTGCTGCTGCGGTTCTG-3' and 5'-CCTCTCCACCATCTGCTTGTAG-3' for plexin B3, 5'-CGCACCACTGGCATTGTCAT-3' and 5'-TTC TCCTTGATGTCACGCAC-3' for β-actin, respectively. The primer sequences were synthesized by Beijing Genomics Institute (China). The PCR products were resolved in 1.5 % agarose gels and visualized by staining with ethidium bromide. To quantify the PCR products, bands representing the amplified products were analyzed by Quantity One Analysis Software (BIO-RAD Co., USA).

Real-time PCR

The reaction mixture volume was made up to 50 μL. Quantitative RT-PCR was performed using SYBR GreenER qPCR SuperMix reagents (Invitrogen) and a Bio-Rad iCycler. Relative transcript quantities were calculated using the ΔΔCt method with β-actin as the endogenous reference gene amplified from the samples. PCR conditions were as follows: an initial melting step at 95℃ for 1 min followed by 35 cycles at 95℃ for 90 s, at 60℃ for 30 s, at 72℃ for 30 s, and a final extension at 72℃ for 10 min. The primers used for RT-PCR are 5'-GGTACTGTTCTAGCGACGGC-3' and 5'-ATACTTGGGTTCGGGGTTGT-3' for semaphorin 5A, 5'-AAAGCCACCGAGGAGCCAGAA-3' and 5'-ACTTGACGGCGATGGGGATG-3' for plexin B3, 5'-TGACGTGGACATCCGCAAAG-3' and 5'-CTGGA AGGTGGACAGCGAGG-3' for β-actin, respectively.

Western blotting

Frozen specimens were homogenized in a lysis buffer [50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 0.25 % sodium deoxycholate, 1 % Triton X-100, 0.1 % sodium dodecyl sulfate (SDS), 1 mmol/L NaF, 1 mmol/L Na_3VO_4], and protease inhibitors (10 mg/L aprotinin and 1 mmol/L phenylmethylsulfonyl fluoride) were added to obtain total protein. An equal amount of protein, quantified with a bicinchoninic acid protein assay kit (Pierce Biotechnology, Rockford, IL, USA), was subjected to 10% SDS-polyacrylamide gel electrophoresis, and transferred to polyvinylidene difluoride membrane. The membranes were blocked with 5 % nonfat milk in Tris buffered saline with Tween 20 [TBST, 50 mmol/L Tris-HCl (pH 7.6), 150 mmol/L NaCl, 0.1 % Tween 20] for 2 h at room temperature, and subsequently incubated with primary anti-rabbit polyclonal antibody (anti-semaphorin 5A diluted at 1:400 and plexin B3 diluted at 1:500 and β-actin diluted at 1:2000 were purchased from Santa Cruz Biotechnology) in a blocking buffer at 4℃ overnight. Following a

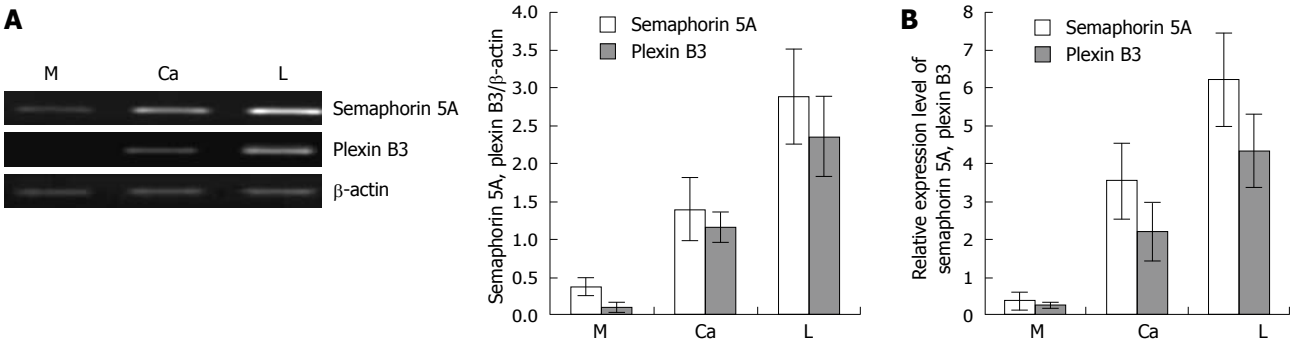

Figure 1 Expression of semaphorin 5A and plexin B3 mRNA in 48 samples of primary gastric carcinoma tissues and its corresponding non-neoplastic mucosa as well as matched regional lymph node metastasis. A: A representative result (left panel) and summary (right panel) of semaphorin 5A and plexin B3 expression in 48 samples of primary gastric carcinoma (Ca) and its corresponding nonneoplastic mucosa (M) as well as matched regional lymph node metastasis (L) examined by RT-PCR. The expression of β-actin was used as an internal control; B: Real time RT-PCR for relative expression levels of semaphorin 5A and plexin B3 in 48 samples of primary gastric carcinoma (Ca) and its corresponding nonneoplastic mucosa (M) as well as matched regional lymph node metastasis (L).

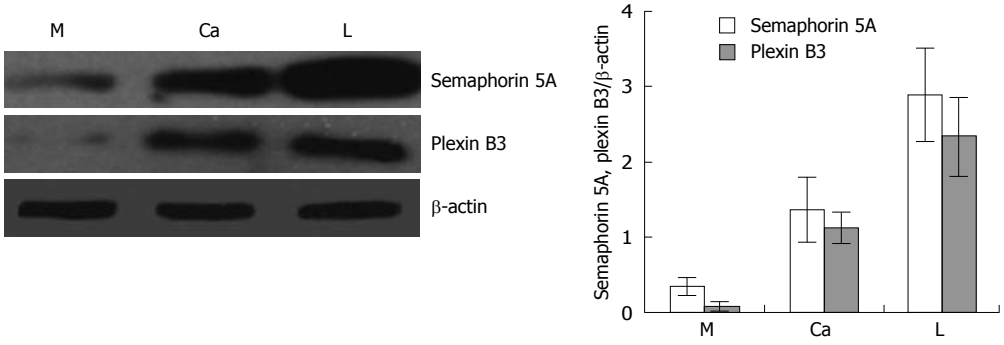

Figure 2 A representative result (left panel) and summary (right panel) of semaphorin 5A and plexin B3 protein expression in 48 samples of primary gastric carcinoma (Ca) and its corresponding nonneoplastic mucosa (M) as well as matched regional lymph node metastasis (L) examined by Western blotting. The expression of β-actin was used as an internal control.

washing with TBST, the membranes were incubated with horseradish peroxidase-conjugated rabbit anti-mouse secondary antibody (1:1000, Dako, Glostrup, Denmark) for 2 h at room temperature. The membranes were washed with TBST, and protein bands were visualized with enhanced chemiluminescence according to its manufacturer's instructions (KPL, Gaithersburg, USA). β-actin bands were taken as a loading control. Protein quantity was analyzed using the UTHSCSA Image Tool 3.0. Target protein expression was evaluated using the relative intensity ratio of target protein/loading control.

Statistical analysis

Results were expressed as mean ± SD. Statistical differences between different groups were assessed by ANOVA using SPSS12.0 statistical software. $P < 0.05$ was considered statistically significant.

RESULTS

Semaphorin 5A and plexin B3 mRNA expression

To infer the status of semaphorin 5A and plexin B3 in the invasion and metastasis of gastric carcinogenesis, we evaluated the mRNA expression of semaphorin 5A and plexin B3 using semi-quantitative RT-PCR in 48 samples of primary gastric carcinoma tissue and its corresponding non-neoplastic mucosa as well as matched regional lymph node metastasis. A representative result of RT-PCR for semaphorin 5A and plexin B3 expression is shown in Figure 1A. The expression of semaphorin 5A and plexin B3 mRNA gradually increased in nonneoplastic mucosa, primary gastric carcinoma and lymph node metastasis ($P < 0.05$). Moreover, the expression of semaphorin 5A was closely correlated with that of plexin B3. To confirm the RT-PCR result of semaphorin 5A and plexin B3, we performed real-time RT-PCR analysis in 20 samples of cDNAs from primary gastric cancer and non-neoplastic mucosa as well as matched regional lymph node metastasis. Similar results were observed (Figure 1B).

Semaphorin 5A and plexin B3 protein expression

The expression levels of semaphorin 5A, and plexin B3 were also measured by Western blotting in primary gastric carcinoma tissue and its corresponding nonneoplastic mucosa as well as matched regional lymph node metastasis. A representative result of Western blotting for the expression of semaphorin 5A and plexin B3 is shown in Figure 2. After normalization with β-actin, the expression of Semaphorin 5A and plexin B3 protein gradually increased in non-neoplastic mucosa, primary gastric carcinoma and lymph node metastasis ($P < 0.05$), which was consistent with the result from RT-PCR analysis.

DISCUSSION

Semaphorin 5A is a member of class 5 semaphorins which are anchored to cell membranes and characterized by seven type 1 thrombospondin repeats. Plexin B3,

belonging to class B plexin subfamily, was identified as a specific and functional receptor for semaphorin 5A. Semaphorin 5A and plexin B3 have been originally characterized as constituents of the complex regulatory system responsible for the wiring of neural networks during the development of the central nervous system, and subsequently found to participate in the activities outside of the nervous system such as migration of neural crest cells and heart development to name but a few examples[11,12]. However, very little is known about the expression and role of semaphorin 5A and plexin B3 in human cancers including gastric carcinoma. A random p-element insertion screen has been used to identify genes that modulate tumor progression and tumorigenicity in Drosophila study[13]. One of the genes identified in this screen is the Drosophila homologue of semaphorin 5C, with which semaphorin 5A shares a high sequence similarity[13]. In addition, experiments performed by Paolo Conrotto and co-workers revealed that semaphorin 5A, through plexin B3, stimulates the tyrosine kinase activity of Met and RON which has been shown to play a role in tumor progression[14,15]. Most importantly, there is cumulative evidence that certain semaphorins and their receptors implicated in axonal path finding in the developing nervous system are expressed in multiple types of cancer cells, modulate the behavior of cancer cells, promote tumor angiogenesis and progression by multiple mechanisms[16]. Taken together, these observations implicate that semaphorin 5A may play a role in the development and progression of human tumors by interacting with plexin B3.

To explore whether semaphorin 5A and plexin B3 are associated with the invasion and metastasis of gastric cancer and exert certain biological functions outside of the nervous system, we investigated the protein and mRNA expression of semaphorin 5A and plexin B3 in primary gastric carcinoma and its corresponding nonneoplastic mucosa as well as matched regional lymph node metastasis by RT-PCR and Western blotting assay. Our experimental results showed that the protein and mRNA expression level of semaphorin 5A was the lowest in normal gastric mucosa, moderate in primary gastric carcinoma, and the highest in lymph nodes with metastatic gastric carcinoma, respectively ($P < 0.05$). In contrast, plexin B3 and semaphorin 5A had a similar expression pattern, suggesting that the expression of plexin B3 is closely correlated with that of semaphorin 5A. These findings demonstrate that the expression of semaphorin 5A and its receptor plexin B3 increased gradually with gastric cancer procession, indicating that semaphorin 5A may play an important role in the invasion and metastasis of gastric carcinoma through plexin B3, displaying a novel expression and function of semaphorin 5A and plexin B3 outside of the nervous system. To our knowledge, this is the first report on the expression of semaphorin 5A and plexin B3 mRNA and protein in gastric carcinoma, and the functional role of semaphorin 5A and plexin B3 in the invasion and metastasis of gastric cancer.

In conclusion, semaphorin 5A and plexin B3 expression increases significantly with gastric carcinoma progression, and semaphorin 5A and plexin B3 may be involved in the processes of gastric cancer invasion and metastasis. Therefore, the novel expression and function of semaphorin 5A and plexin B3 outside of the nervous system not only add more knowledge about semaphorin 5A and plexin B3, but also shed some lights on the pathogenesis of gastric carcinoma, and probably represent a new therapeutic target for gastric carcinoma.

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COMMENTS

Background

Gastric carcinoma is one of the most common malignant tumors in China. Invasion and metastasis are the main cause of cancer-related death. Therefore, it is necessary to investigate the mechanism underlying invasion and metastasis of malignant tumors. Semaphorin 5A and its receptor plexin B3 have been originally described in the nervous system, and are important in axon migration and proper central nervous system development. However, very little is known about the expression and role of semaphorin 5A and plexin B3 in human cancers including gastric carcinoma.

Research frontiers

Experiments were performed to study the expression of semaphorin 5A and plexin B3 in gastric carcinoma and its relation with tumor invasion and metastasis. This study showed that the expression of semaphorin 5A and plexin B3 increased gradually in non-neoplastic mucosa, primary gastric carcinoma, and lymph node metastasis, suggesting that the expression of semaphorin 5A and plexin B3 is closely correlated to the invasion and metastasis of gastric cancer.

Innovations and breakthroughs

This is the first report on the expression of semaphorin 5A and plexin B3 in gastric carcinoma, and the relation of semaphorin 5A and plexin B3 with the invasion and metastasis of gastric cancer.

Peer review

The authors studied the expression of semaphorin 5A and plexin B3 in gastric carcinoma and its relation with tumor invasion and metastasis, and showed that the expression level increased gradually in non-neoplastic mucosa, primary gastric carcinoma, and lymph node metastasis, and was positively related to tumor invasion and metastasis, which can be used in research of gastric carcinoma, and provide a new target for gastric carcinoma treatment.

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CASE REPORT

Ligation-assisted endoscopic mucosal resection of gastric heterotopic pancreas

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Abstract

Heterotopic pancreas is a congenital anomaly characterized by ectopic pancreatic tissue. Treatment of heterotopic pancreas may include expectant observation, endoscopic resection or surgery. The aim of this report was to describe the technique of ligation-assisted endoscopic mucosal resection (EMR) for resection of heterotopic pancreas of the stomach. Two patients (both female, mean age 32 years) were referred for management of gastric subepithelial tumors. Endoscopic ultrasound in both disclosed small hypoechoic masses in the mucosa and submucosa. Band ligation-assisted EMR was performed in both cases without complications. Pathology from the resected tumors revealed heterotopic pancreas arising from the submucosa. Margins were free of pancreatic tissue. Ligation-assisted EMR is technically feasible and may be considered for the endoscopic management of heterotopic pancreas.

Key words: Endoscopic mucosal resection; Endoscopic ultrasound; Heterotopic pancreas

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INTRODUCTION

Heterotopic pancreas (HP), also known as pancreatic rest, was first described in 1727 when it was found in an ileal diverticulum[1]. It refers to an uncommon congenital anomaly characterized by the presence of ectopic pancreatic tissue far from the pancreas and without any anatomical or vascular communication with this organ. It occurs in 2% of the general population[2] and is more common in males than females[3]. HP may occur throughout the gastrointestinal tract but has a proclivity for involving the stomach and proximal small intestine. Most affected patients are asymptomatic although a minority may present with a variety of symptoms, most common being epigastric pain[2]. Options for treatment for heterotopic pancreas in the stomach include surgery[2-4], endoscopic resection[5-8] or conservative management. This report describes the first two cases of HP treated with band ligation-assisted endoscopic mucosal resection (EMR).

CASE REPORT

Case 1

A 49-year-old white female presented to her primary gastroenterologist with abdominal bloating and constipation. She was report anemic and found to have heme-occult positive stools. Work-up included a computer tomography (CT) scan of the abdomen and pelvis, colonoscopy and esophagogastroduodenoscopy (EGD). CT revealed small liver cysts but no other abnormalities. Colonoscopy demonstrated two small polyps that were removed. EGD showed a small, firm, umbilicated subepithelial antral mass. The mucosa overlying the mass was biopsied and showed chronic gastritis with no submucosal tissue present. The patient was referred to our endoscopy unit for upper endoscopic ultrasonography (EUS) of the antral lesion.

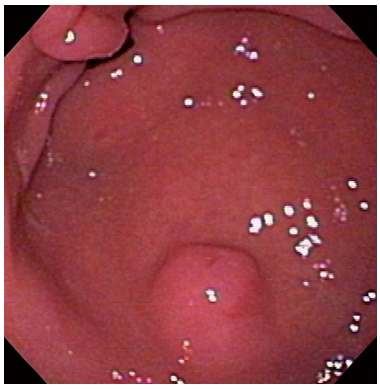

Figure 1 Endoscopic view of heterotopic pancreas of the stomach showing a medium-sized subepithelial nodule with central umbilication and normal overlying mucosa. The pylorus is visible distally.

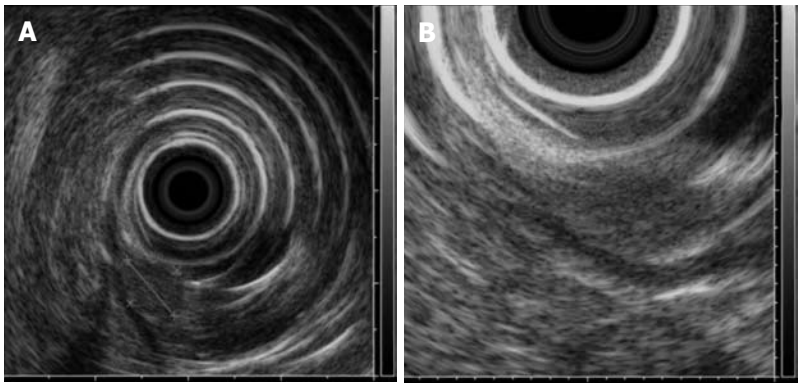

Figure 2 Radial EUS images. A pancreatic rest showing an 8 mm × 6 mm hypoechoic subepithelial mass appearing to involve the mucosa (A) and submucosa (B). The tumor was confirmed as submucosal in origin after resection without involvement of the mucosa.

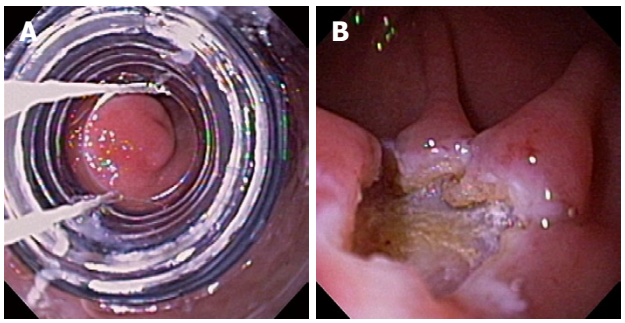

Figure 3 Ligation-assisted EMR of pancreatic rest. The banding device was positioned over the target lesion (A), suction was applied and a band was deployed. The lesion was then resected using electrocautery snare. Residual ulcer is shown (B).

She denied abdominal pain, nausea, vomiting, overt gastrointestinal bleeding or weight loss. Her physical examination revealed a healthy middle-aged white female in no apparent distress. Her abdominal examination was normal without tenderness. Prior to EUS, she was informed that the lesion most likely represented a pancreatic rest and that if suspected diagnosis was confirmed, then conservative management would be reasonable. Nevertheless, the patient expressed her preference for resection of the mass if possible. Informed consent was obtained and sedation was performed using a combination of intravenous midazolam and propofol. Initial EGD confirmed a single medium-sized subepithelial nodule along the greater curvature of the gastric antrum (Figure 1). The patient was then placed into the right lateral decubitus position and water was instilled into the distal stomach. Radial endosonography (GF-UE160-AL5; Olympus America Inc., Center Valley PA; USA) revealed a well-defined hypoechoic lesion from the deep mucosa/submucosa that measured 8 mm × 6 mm in maximal diameter (Figure 2A and B). There was no evidence of peritumoral adenopathy. Ligator-assisted EMR (Duette Multi-Band Mucosectomy; Cook Medical Inc., Winston-Salem, NC; USA) of the tumor was successfully performed using one band (Figure 3). The residual ulcer was closed using three endoclips (Resolution Clip; Boston Scientific Inc., Natick MA; USA). There were no complications. Surgical pathology from the resected specimen revealed heterotopic pancreatic tissue confined to the submucosa (Figure 4).

Case 2

A 15-year-old white female presented to her primary gastroenterologist with left upper quadrant abdominal pain. EGD showed a small subepithelial antral mass. The mucosa overlying the tumor was normal and biopsies showed only mild gastritis. The patient was then referred for an upper EUS. She denied nausea, vomiting, overt gastrointestinal bleeding or weight loss. Physical examination including abdominal examination was normal. Prior to EUS, review of endoscopic pictures from outside EGD suggested a pancreatic rest. Information was provided to the patient and parents that if suspected diagnosis was confirmed, then conservative management would be reasonable. Nevertheless, the patient and parents both expressed preference for tumor resection, if possible. Initial EGD confirmed a single medium-sized subepithelial nodule along the greater curvature of the prepyloric stomach (Figure 5). The patient was then placed into the right lateral decubitus position and water was instilled into the distal stomach. Radial endosonography revealed a well-defined 9 mm × 7 mm hypoechoic lesion from the deep mucosa and submucosa (Figure 6). The outer endosonographic borders were well defined. Ligator-assisted EMR of the tumor was successfully performed using one band (Figure 7). There were no complications. The resected specimen was submitted for pathologic examination, which revealed the presence of HP tissue confined to the submucosa with negative margins.

DISCUSSION

Heterotopic pancreas (HP), also known as pancreatic rest, is most often detected as an incidental finding during routine upper endoscopy. The typical endoscopic appearance in the stomach is as a firm round or oval umbilicated subepithelial nodule along the greater curvature situated

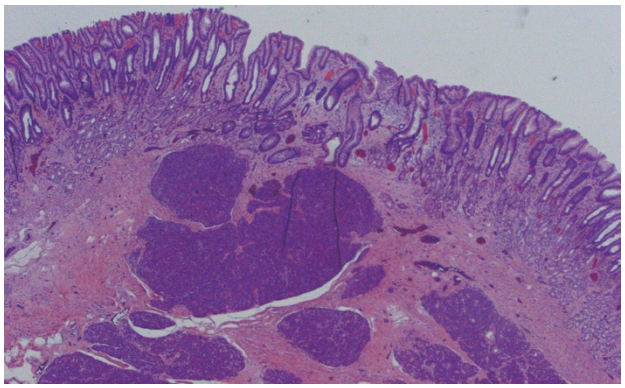

Figure 4 Photomicrograph of the resection specimen showing pancreatic tissue within the gastric submucosa (HE, × 10).

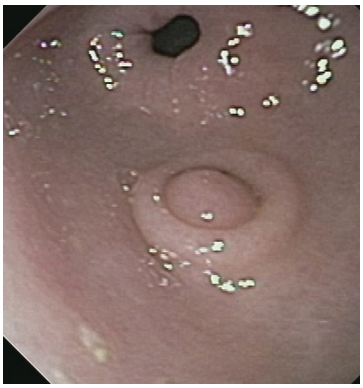

Figure 5 Endoscopic view of heterotopic pancreas of the stomach showing a medium-sized subepithelial nodule with central umbilication and normal overlying mucosa. The pylorus is visible distally.

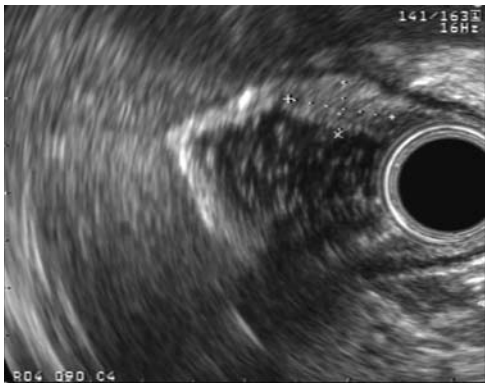

Figure 6 Radial EUS images of a pancreatic rest showing a 9 mm × 7 mm hypoechoic subepithelial mass appearing to involve the deep mucosa and submucosa. The tumor was confirmed as submucosal in origin after resection without involvement of the mucosa.

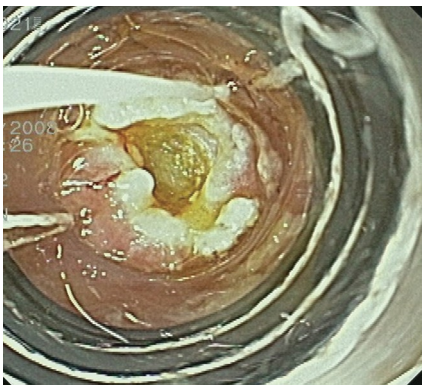

Figure 7 Ligation-assisted EMR of pancreatic rest.

several centimeters proximal to the pylorus. Most patients are asymptomatic, but symptoms may rarely occur due to the irritative effect of the hormones and enzymes secreted by the HP at a particular site[3]. Histologically, pancreatic rests vary from resembling the normal pancreas (acini, ducts and islets of Langerhans) to widely separated ducts within a muscular stroma[4,9]. Hence, complications in HP resemble those seen with the pancreas itself including acute pancreatitis[10,11], pancreatic cancer[12], cystic degeneration[13] and islet cell tumors[14]. Other rare reported complications include gastrointestinal bleeding[15], gastric outlet obstruction[16], jaundice[13] and abscess formation[10]. Asymptomatic HP can generally be followed expectantly with treatment reserved for patients who are symptomatic, have enlarging lesions or to ensure diagnostic certainty. Both pancreatic rests in these two patients were incidentally discovered during EGD for investigation of anemia and left upper quadrant pain, respectively. Nevertheless, removal was requested by both patients to ensure the suspected pre-procedural diagnosis.

The diagnosis of HP is not straightforward. Although umbilication is characteristic, the specificity of this endoscopic finding for HP remains unknown. The typical EUS image of a pancreatic rest in the stomach is a hypoechoic, heterogeneous submucosal mass, although the muscularis propria and mucosa may be occasionally involved. A ductal structure may also be discernible within the lesion. Although these endosonographic findings are suggestive of HP, the accuracy of EUS for the diagnosis of subepithelial tumors (other than gastrointestinal stromal tumors such as HP) is limited[7,17]. This fact is illustrated in our cases as the mucosa was incorrectly predicted to be involved in both patients.

Since HP is usually a submucosal tumor, pinch mucosal biopsies are invariably non-diagnostic. Nevertheless, Shalaby *et al*[18] reported a case of esophageal HP diagnosed with biopsies obtained with a jumbo forceps. Teixeira[19] has described the use of ethanol injection to create an artificial ulcer that then facilitated the removal of sufficient submucosal tissue to establish the diagnosis of HP. Goto *et al*[20] reported a case of esophageal HP diagnosed by EUS-guided FNA. Historically, the diagnosis of HP was often made by histological examination of surgical specimens. Some authors believe that it is difficult to obtain a definitive diagnosis of HP preoperatively[21] and therefore advocate surgical resection[3].

Although there are some reports of surgical resection of HP[2-4], EMR may be an attractive, less invasive option for resection of accessible lesions. However, there have only been a few reports[5-8] that describe the use EMR for HP resection (Table 1). Two groups have described the use of cap-assisted EMR[5,6]. The "inject, lift and cut", or "strip biopsy" EMR technique[7] was reported for the resection of 6 HP submucosal masses. Finally, Sun *et al*[8]

Table 1 Literature summary of HP cases treated with EMR

Author	Yr	Number of tumors	Mean tumor size (mm)	Tumor location	EMR technique	Follow-up[1] (mo)
Lee *et al*[5]	1999	1	Small	Stomach	CAP-assisted	NR
Faigel *et al*[6]	2001	1	10	Stomach	CAP-assisted	6
Kojima *et al*[7]	1999	6	13.5	Stomach	Strip biopsy	15
Sun *et al*[8]	2002	2	NR	Stomach	Inject and cut	12-17
Khashab *et al* (current study)	2008	2	8.5	Stomach	Ligation-assisted	NR

EMR: Endoscopic mucosal resection; NR: Not reported. [1]All cases with reported follow-up were without any recurrence.

studied the use of EUS-guided injection and the "inject and cut" EMR technique for the resection of 16 upper gastrointestinal submucosal tumors, two of which were gastric HP tumors. Ligation-assisted EMR may be more operator-friendly than the other EMR techniques. It requires neither saline injection nor snare prepositioning, and the concept of tissue capture is similar to the familiar variceal ligation technique. The current series, to our knowledge, is the first to describe the use of ligation-assisted EMR for gastric HP. This technique permitted a histologic confirmation of the suspected diagnosis and in both cases achieved a margin-negative resection without complication.

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CASE REPORT

Meckel's diverticulum masked by a long period of intermittent recurrent subocclusive episodes

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Abstract

Meckel's diverticulum (MD) is the most frequent congenital abnormality of the small bowel and it is often difficult to diagnose. It is usually asymptomatic but approximately 4% are symptomatic with complications such as bleeding, intestinal obstruction, and inflammation. The authors report a case of a 7-year-old boy with a one-year history of recurrent periumbilical colicky pain with associated alimentary vomiting, symptoms erroneously related to a cyclic vomiting syndrome but not to MD. The clinical features and the differential diagnostic methods employed for diagnosis of MD are discussed.

Key words: Meckel diverticulum; Abdominal pain; Recurrent subocclusive episodes, Diagnostic imaging

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INTRODUCTION

Meckel's diverticulum (MD) occurs in about 2% of the population, making it the most prevalent congenital abnormality of the gastrointestinal tract. It can be asymptomatic or mimic common abdominal disorders. We report a case of a child with an intraoperative diagnosis of MD, with a long history of recurrent abdominal pain and vomiting misdiagnosed as a cyclic vomiting syndrome.

CASE REPORT

A 7-year-old boy was referred with a one-year history of periumbilical colicky pain with associated alimentary vomiting. The frequency of the episodes increased from one per month to weekly and then daily vomiting. The pain usually spontaneously disappeared within a few hours. During the weeks before our visit, the painful episodes lasted longer and were reported to occur also at night. The child lost one kilogram in one month. No diarrhoea was reported, the boy was rather constipated. Previous medical investigations, abdominal ultrasonography and plain abdominal film were negative.

Neither abdominal tenderness, nor liver or spleen enlargement, nor abdominal masses were identified at palpation. Intestinal bacterial overgrowth and celiac disease were excluded by laboratory tests. Complete blood cell count, electrolytes, glycemia, blood ammonia, renal and hepatic function, pancreatic enzymes, C-reactive protein, erythrocyte sedimentation rate, and gamma globulins were within normal ranges.

A plain abdominal film was unremarkable, and a small bowel enema indicated normal transit and normal appearance of the intestinal loops. An abdominal ultrasound (US) revealed a supravesical anechoic mass of about 4 cm × 3 cm × 2 cm, with fluid inside (Figure 1).

Pelvic magnetic resonance imaging (MRI) confirmed the presence of the 4 cm mass, located above and behind the bladder, slightly to the right of the midline, with liquid content (Figure 2).

With the suspicion of a mesenteric cyst or an intestinal duplication, the child underwent an exploratory laparoscopy. The intraoperative macroscopic finding was that of MD. Diagnosis was confirmed by histological features.

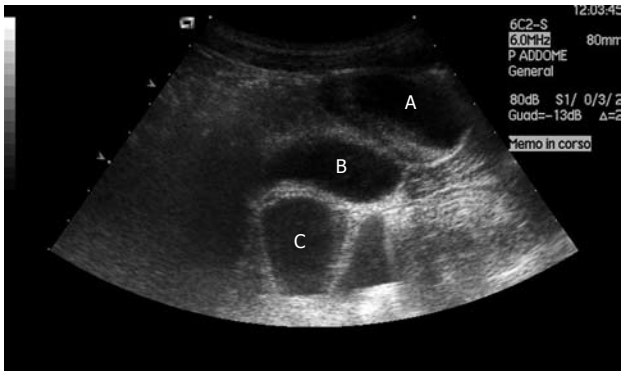

Figure 1 Abdominal ultrasound: anechoic oval supravesical lesion with fluid-fluid level (A); bladder (B); bowel (C).

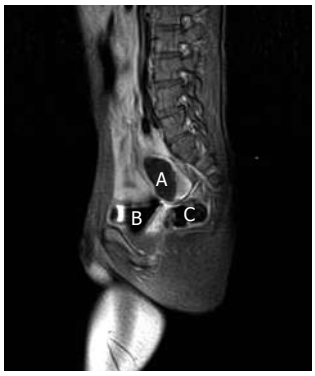

Figure 2 Abdominal MRI; T1 weighted sequence with fat-suppression (SPIR) after contrast medium, saggital plane: hypointense oval supravesical lesion with air-fluid level and thin enhanced wall (A); bladder (B); rectum (C).

DISCUSSION

MD is the most common congenital anomaly of the gastrointestinal tract. The "rule of two" can remind us of some of its main features: occurs in 2% of the population; usually discovered before 2 years of age; occurs within 2 feet of the ileocecal valve; is 2 inches long and 2 cm in diameter[1]. It is the result of an incomplete atrophy of the omphalomesenteric duct. The location of the diverticulum is on the antimesenteric border of the small intestine, most frequently between 30 cm and 90 cm from the ileocecal valve; there can be a fibrous connection to the umbilicus, as the remnant of the partially obliterated vitelline duct.

MD is a true diverticulum, composed of all layers of the intestinal wall, and is lined by normal small intestine epithelium. Gastric heterotopias can be found in roughly 50% of cases, and pancreatic, duodenal, colonic, or biliary mucosa have rarely been reported.

MD can be silent all through a lifetime: clinical symptoms arise from complications (MD carriers have a 4% lifetime risk of developing a complication)[2]. Hemorrhage is the result of peptic ulceration of the ileal mucosa next to an acid-producing gastric mucosal heterotopia: the presentation of the blood loss varies from recurrent minimal intestinal bleeding, to a massive, shock-producing hemorrhage, and it is usually painless. Diverticulitis can mimic an acute appendicitis: pain is frequently localized in the midline or slightly to the right and, as in appendiceal disease, inflammation can progress until perforation. The diverticulum can invert into the ileal lumen and become the starting point of an ileo-ileal or ileo-ileo-colic intussusception: symptoms can not be discriminated from those ascribed to idiopathic intussusception, even though the onset of the former is described to occur at an earlier age. A further mechanism by which MD can produce intestinal obstruction is to turn around a fibrous remnant: symptoms may vary from intermittent recurrent subocclusive episodes, as in our patient, to frank occlusion with strangulation features if a complete volvulus occurs[3]. "Littre hernia" occurs when a MD protrudes into a potential abdominal opening such as umbilical, inguinal, or femoral, and can be accompanied in some cases by entrapment, inflammation, and necrosis[1]. Tumors are reported in 0.5% to 3% of symptomatic diverticula in adulthood (carcinomas in one third of the cases)[3].

Preoperative diagnosis of a complicated MD can be challenging and often difficult to establish because clinical symptoms and imaging features overlap with those of other disorders causing acute abdominal pain or gastrointestinal bleeding[4].

Initially, our case was misdiagnosed as a cyclic vomiting syndrome and a functional abdominal pain, since neither inflammatory nor bleeding clinical features were present, and laboratory tests were substantially within normal ranges. If any bleeding episode had been reported, a 99mTc-pertechnetate scintigraphy would have been indicated: the principle is that a bleeding diverticulum consists of ulcerated ectopic gastric mucosa that can be revealed with 99mTc-pertechnetate. This concentrates in gastric tissue leading to a reported sensitivity of between 60% and 80%[5].

The progressive worsening of painful episodes, however, prompted us to exclude causes of pain and vomiting requiring surgery, such as intestinal malrotation, which was ruled out by a normal small bowel enema. It is reported in the literature that enteroclysis may be of help in detecting MD but in our case the diverticular image was missed[6].

The US findings of a cyst supported the suspicion of a surgery-indicated cause for the painful episodes, but other gut malformations such as mesenteric cysts or enteric duplications, both of which can present with subocclusive symptoms, were taken into account in the differential diagnoses of our child. US and computed tomography are reported in the literature to be valuable radiological investigations in MD patients without the classical history of painless hemorrhage[7,8].

Complicated MD has a spectrum of radiological features which may help in the preoperative investigations, but are not always diagnostic[7-9]. Our rationale in choosing MRI as a second-line radiological examination lay in the fact that we needed a better anatomical definition of what we suspected was a pelvic mass, without further irradiation of the child: there is no evidence for the use of MRI to detect MD in the literature. Final diagnosis is almost always done at surgery: exploratory laparoscopy is recommended because it affords the possibility of simultaneous surgical resection, which is the definitive cure of a symptomatic MD[10].

In conclusion, Although MD is the most prevalent congenital abnormality of the gastrointestinal tract, it is often difficult to diagnose. The diagnosis of MD should be considered in children with intestinal bleeding, unexplained recurrent abdominal pain, and nausea and vomiting suggestive of cyclic vomiting syndrome.

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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
London, UK
Gastro 2009 UEGW/World Congress of Gastroenterology
www.gastro2009.org

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Chinese journal article (list all authors and include the PMID where applicable)

2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension,

insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 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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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

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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 billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

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

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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

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

Statistical data

Write as mean ± SD or mean ± SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

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Use SI units. For example: body mass, m (B) = 78 kg; blood pressure, p (B) = 16.2/12.3 kPa; incubation time, t (incubation) = 96 h, blood glucose concentration, c (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, p (CEA) = 8.6 24.5 μg/L; CO_2 volume fraction, 50 mL/L CO_2, not 5% CO_2; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc*. Arabic numerals such as 23, 243, 641 should be read 23 243 641.

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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*.
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EDITORIAL

Drug-induced liver injury: Is it somehow foreseeable?

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Abstract

The classic view on the pathogenesis of drug-induced liver injury is that the so-called parent compounds are made hepatotoxic by metabolism (formation of neo-substances that react abnormally), mainly by cytochromes P-450 (CYP), with further pathways, such as mitochondrial dysfunction and apoptosis, also playing a role. Risk factors for drug-induced liver injury include concomitant hepatic diseases, age and genetic polymorphisms of CYP. However, some susceptibility can today be predicted before drug administration, working on the common substrate, by phenotyping and genotyping studies and by taking in consideration patients' health status. Physicians should always think of this adverse effect in the absence of other clear hepatic disease. Ethical and legal problems towards operators in the health care system are always matters to consider.

Key words: Drug-induced liver injury; Cytochrome P-450; Drug metabolism; Pharmacogenomics; Herbal remedies

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INTRODUCTION

Drug metabolism is the major determinant of drug clearance and the factor most frequently responsible for inter-individual differences in drug pharmacokinetics. Most adverse hepatic reactions require metabolism of the drug to form reactive metabolites and free radicals (indirect toxicity), that subsequently lead to fatal insults, sensitization to the lethal effects of the innate immune system, or haptenization eliciting an immunoallergic response of the adaptive immune system. Besides licensed drugs, herbal and natural supplements are recognized as causing hepatotoxicity with increasing frequency as patients turn more and more to alternative medicine[1].

Many environmental and developmental factors can interact with each other and with genetic factors to affect drug response and the metabolic activation or inactivation of drugs generally used in medical practice.

Oxidation, reduction and hydrolysis are the main pathways along drug metabolism before excretion. The most important enzyme system of phase I metabolism is the cytochrome P-450 (CYP) system, a microsomal superfamily of isozymes that catalyze the oxidation of many drugs. The electrons are supplied by NADPH/CYP reductase, a flavoprotein that transfers electrons from NADPH (the reduced form of nicotinamideadenine dinucleotide phosphate) to CYP. Multiple forms of CYP enzymes play important roles in the oxidation of structurally diverse xenobiotics. CYP3A (about 30% of total CYP) and CYP2C (about 20% of total CYP) enzymes are major forms. Human cytochrome CYP3A subfamily members (mainly CYP3A4) mediate the metabolism of many marketed drugs (amiodarone, amlodipine, clarithromycin, cyclosporine, erythromycin, lovastatin, nifedipine, tamoxifen, terfenadine, verapamil, R-warfarin) and thus play a critical role in drug metabolism. Furthermore, P-glycoprotein and CYP3A are frequently co-expressed in the same cells and share a large number of substrates and modulators[2].

Other important human drug-metabolizing enzymes are CYP1A2 (caffeine, estradiol, lidocaine, tacrine, theophylline, verapamil, R-warfarin), CYP2C9 (diclofenac, phenytoin, piroxicam, tetrahydrocannabinol, tolbuta-

mide, S-warfarin), CYP2C19 (diazepam, hexobarbital, S-mephenytoin, omeprazole, pentamidine, propranolol, R-warfarin), and CYP2D6 (codeine, debrisoquine, dextromethorphan, encainide, haloperidol, metoprolol, mexiletine, paroxetine, phenothiazines, propanolol, risperidone, sertraline, tricyclic antidepressants, venlafaxine) as highlighted by commonly used probe cocktails[3,4]. The expression of drug metabolizing enzymes, mainly CYP, shows significant interspecies differences and variability among human individuals (polymorphic or inducible enzymes) which makes the accurate prediction of the metabolism of various compounds in humans difficult. For example, patients who metabolize certain drugs rapidly may require higher, more frequent doses to achieve therapeutic concentrations; patients who metabolize certain drugs slowly may need lower, less frequent doses to avoid toxicity, particularly for drugs with a narrow interval of safety.

HEPATOTOXICITY: GENERAL CONCEPTS

Several key issues need to be addressed to study drug induced liver injury (DILI), that is, what metabolites will be formed (metabolic profile); which enzymes are involved and to what extent; whether drug metabolism will be affected directly (drug-drug interactions) or indirectly (enzyme induction) by the administered compound. Drug metabolism studies are routinely performed in laboratory animals, but they are not sufficiently accurate to predict the metabolic profiles of drugs in humans. In fact, hepatotoxicity due to idiosyncratic reactions cannot be detected by conventional animal toxicity studies. Furthermore, predisposing factors in humans such as ethanol-induced metabolic sensitivity to acetaminophen hepatotoxicity do not exist in the animal model. Interspecies differences in bioavailability, distribution and metabolism may also explain a number of false positives and false negatives. There is clearly a need to better understand the drug metabolism enzyme profile of the most commonly used non-rodent species, i.e. the dog and the monkey. Some false negative results may be related to insufficient (not in the pharmaceutical industry) exposure of the animals either because the doses tested were too low or because intestinal absorption was poor. Many of these issues can now be addressed by the use of relevant human *in vitro* models, which may speed up the understanding of drug toxicity. Human hepatocytes are the closest *in vitro* model to the human liver and they are one of the few models which can produce a metabolic profile of a drug which is very similar to that found *in vivo*. However, the use of human hepatocytes is restricted, because limited access to suitable tissue samples prevents their use in high-throughput chemical and genetic screens[5]. Comparative studies on liver microsomes and cells from animal species, including humans, are very useful for demonstrating species differences in the metabolic profile of a given drug and are of great value in the selection of animal species for later pharmacokinetic and toxicological studies[6].

CYP-engineered cells (or microsomes from CYP-engineered cells, for example, Supersomes) have made the identification of CYPs involved in the metabolism of a drug candidate more straightforward and much easier[7]. However, the screening of substances acting as potential CYP inducers can be conducted only in cellular systems fully capable of transcribing and translating CYP genes.

EPIDEMIOLOGY AND CLINICAL ASPECTS OF DRUG INDUCED LIVER INJURY

Drug hepatotoxicity has been evaluated in case histories, surveys based on retrospective record reviews, and spontaneous adverse drug reactions reported to national pharmacovigilance systems, but in relatively few epidemiologic studies. Approximately 1 in 100 patients develops DILI during hospitalisation. DILI is frequently missed and, therefore, DILI detection by diagnosis will result in misleadingly low incidence rates[8]. Unfortunately, patients with drug-induced hepatocellular jaundice have an 11.7% chance of progressing to death or transplantation[9].

DILI cases have been reported to constitute approximately 6% of all out-patients and 3% of referrals and to occur more often in women[10]. The incidence of drug-induced hepatitis is higher in patients over 40 years of age[11]. Acute liver failure or injury not clearly attributable to other known causes occurred in the order of 1 per 10 000 person -years among diabetic patients treated with oral hypoglycemic drugs or insulin[12].

The long term outcome of drug-related liver disease is unknown. To study the natural history of histologically proved drug-induced hepatotoxicity, 44 patients with liver biopsies coded as drug-related liver disease were identified from hospital records. Initial histology showed acute hepatitis in 6, chronic hepatitis in 20, and cholestasis in 18. At a median of 5 years follow-up, one third of patients had persistent significant abnormalities in their liver blood tests. Factors predicting persistence or development of chronic liver disease were fibrosis and continued exposure to the drug[13]. To identify and quantify the risk of acute liver injury associated with individual drugs, authors reviewed and integrated all the published epidemiologic research on the subject. Participants were selected according to their use of selected agents [nonsteroidal antiinflammatory drugs (NSAIDs), antibiotics, acid-suppressing drugs, other drugs suspected of being hepatotoxic] during the study period. Among the agents, authors found a group of important hepatotoxic drugs, including chlorpromazine and isoniazid, with an associated incidence rate of acute liver injury greater than 100/100 000 users. Agents with less risk but greater than 10/100 000 users were amoxicillin-clavulanic acid and cimetidine[14].

DILI with an incidence rate of near 1 per 100 000 encloses a spectrum of clinical disease ranging from no symptoms with mild biochemical abnormalities to fatal, fulminant hepatitis. The majority of adverse liver reactions are idiosyncratic in nature; in fact, about 10% of all acute liver failure cases are attributed to this type of reaction. They can occur in some instances up to three months after the causative medication was last taken.

The diagnosis of DILI is prevalent clinically, and based primarily on history, that is, exclusion of other hepatic diseases (hepatitis A, B, C, Epstein-Barr virus, cytomegalovirus, ischemia, and biliary tract disease), high likelihood of suspicion based on a strict cause-effect sequence, the duration of latency to symptomatic presentation, the presence of immune-mediated hypersensitivity (hypereosinophilia, fever and rush) and the response to drug withdrawal. Re-challenge is not advised in cases with a hypersensitivity basis, although this is the most definitive means of diagnosis. Some of the hypersensitivity cases are associated with autoantibodies to CYP, which can be used to confirm the diagnosis; for example, halothane is associated with anti-CYP2E1, anticonvulsants are associated with CYP3A4, dihydralazine hepatitis is associated with anti-CYP1A2, and tienilic acid (a diuretic drug withdrawn from the market because of hepatic failure) is associated with anti-CYP2C9. In the cases of metabolic idiosyncrasy, careful reintroduction of the offending drug may be accomplished without recurrence of the liver disease but should be done only when the drug is absolutely necessary and with careful monitoring. In some instances, liver biopsy can be of help, showing characteristic features, but it usually is not necessary and tells more about prognosis than etiology.

DILI can be of hepatocellular (increase of both the transaminases, alanine-aminotransferase/aspartate-aminotransferase), cholestatic (predominant rise in serum alkaline phosphatase and/or γ-glutamyl-transferase, GGT) or mixed type. Negative prognostic factors are an elevated serum bilirubin level and high ammonia levels with a mortality of approximately 10%[15]. A further less favourable index is a marked hypoprothrombinemia. Overall, chronic liver injury may occur in up to 5%-6% of the patients on some drugs, even though the putative offending substance is withdrawn[16].

LIVER HISTOLOGY

As previously emphasized, the pathogenesis of drug- or toxin- induced liver injury usually involves the participation of "toxic metabolites" that either elicit an immune response or directly affect the biochemical processes or functions of the cell[17]. Although the same basic process determines the DILI appearance, the histopathological liver changes in these cases vary, including: (a) necrosis, which commonly occurs in acinar zone 3, (b) abundant neutrophil and/or eosinophil infiltration, (c) hepatocytic and/or canalicular cholestasis with little or no inflammation, (d) microvesicular steatosis mixed with macrovesicular steatosis, and (e) presentation of epitheloid cell granuloma. There are no significant differences in liver histopathology between acute and chronic DILI groups, except that the fibrosis and the ductular proliferation are different.

DAMAGE MECHANISMS

Drug metabolites can be free radicals or electrophilic chemicals that undergo or promote a variety of chemical reactions, such as the depletion of reduced glutathione; covalent binding to proteins, lipids, or nucleic acids; or induction of lipid peroxidation. All of these have consequent direct effects on cellular organelles such as mitochondria, the endoplasmic reticulum, the cytoskeleton, microtubules, or on the nucleus. They may also indirectly influence cellular structures through the activation and inhibition of signaling kinases, transcription factors, and gene-expression profiles. The subsequent intracellular stress leads to cell death caused by either cell shrinkage and nuclear disassembly (apoptosis) or swelling and lysis (necrosis). Liver injury is characterized by hepatocyte death; that is the main event, although bile duct epithelium or sinusoidal endothelial cells may also be involved.

CYP-mediated Biotransformation: the common substrate

While several CYPs are involved in the synthesis of bile acids and steroid hormones and the metabolism of fatty acids, retinoic acid, prostaglandins and eicosanoids, a limited number of CYPs (15 in humans) are primarily involved in xenobiotic metabolism. The xenobiotic-metabolizing CYPs are found in families 1-4. Since a single CYP can metabolize a large number of structurally diverse compounds these enzymes can collectively metabolize chemicals found in the diet, environment and administered as drugs. While metabolism of xenobiotics such as drugs is required to efficiently eliminate them from the body, as noted earlier, certain chemicals are metabolically activated to reactive derivatives that cause cell toxicity and cancer. Among these, the CYPs that metabolically activate toxicants and carcinogens are limited to some forms including CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2C9, CYP2E1, and to a limited extent the CYP3A subfamily.

ATP depletion

Acetaminophen overdose causes liver injury by mechanisms involving glutathione depletion, oxidative stress and mitochondrial dysfunction. Acetaminophen-induced decreases in mitochondrial reduced glutathione and ATP content, and cytosolic leakage of cytochrome c are attenuated by cyclosporin A, suggesting that mitochondrial oxidative stress and ATP depletion resulting from mitochondrial permeability transition (MPT) are the principal mechanisms involved in acetaminophen-induced liver injury[18].

The role of ATP-depletion-dependent necrosis has further been ascertained. Recently, using TUNEL labeling and caspase 3 activation, it was observed that acetaminophen induced the MPT and ATP-depletion-dependent necrosis or caspase-dependent apoptosis as determined, in part, by ATP availability from glycolysis[19].

Binding to nuclear or cytoplasmatic constituents

Inducers can increase the bioactivation of drugs that contribute to hepatotoxicity *via* reactive intermediates. Nuclear receptors are key mediators of drug-induced changes in the expression of drug clearance pathways. However, species differences in nuclear receptor activation make the

prediction of CYP induction in humans from data derived from animal models problematic. Drugs have the capacity to alter nuclear receptor expression (modulators) and/or serve as ligands for the receptors (agonists or antagonists), and thus can have synergistic or antagonistic effects on the expression of drug-metabolizing enzymes and transporters. Co-administration of drugs that are nuclear receptor agonists or antagonists can lead to severe toxicity, a loss of therapeutic efficacy or an imbalance in physiological substrates, providing a novel molecular mechanism for drug-drug interactions[20].

RNA interference

Authors suggested that the lack of p53 response may confer a growth advantage on preneoplastic hepatocytes and may be an important factor in hepatic tumor promotion by 2-acetylaminofluorene and other genotoxic compounds. Inhibition of RNA polymerase Ⅱ driven transcription by DNA lesions may constitute one of the mechanisms leading to accumulation of the tumor suppressor p53[21].

Oxidative stress and lipid peroxidation

Redox signals are important in the modulation of cell function. Reactive oxygen species (ROS) generation influences many signaling proteins, interfering with molecular and biochemical processes responsible for cell differentiation, proliferation and death. Protein kinase (PKC) is a crucial signaling protein which is subject to redox regulation and controls these responses. ROS are products of normal cellular metabolism and are recognized to be harmful or beneficial to living systems. The harmful effect of ROS is termed oxidative stress and it occurs when there is an overproduction of these species or a deficiency of antioxidants. Enzymatic systems that contribute to ROS formation in the liver include CYP monooxygenases and NADPH oxidase. In the healthy liver, hepatocytes produce low amounts of ROS and Kupffer cells are well equipped to release ROS in response to infection. Antioxidants such as superoxide dismutase and catalase efficiently remove ROS surplus to maintain the normal cell homeostasis. Different stimuli are able to modify the redox state of liver cells modulating signal transduction pathways able to trigger many aspects of liver pathologies. In a recent study, evidence of the central role of PKC as a redox-sensitive molecule implicated in the pathogenesis and progression of toxic liver diseases was provided[22].

The free radicals initiate lipid peroxidation by attacking polyunsaturated fatty acids in membranes, setting off a free radical chain reaction sequence. Lipid peroxidation is known to cause membrane disruption, resulting in the loss of membrane integrity and leakage of microsomal enzymes. By-products of lipid peroxidation include reactive aldehydes that can form protein and DNA adducts and may contribute to hepatotoxicity and carcinogenicity, respectively. Natural antioxidants, including glutathione, are capable of quenching the lipid peroxidation reaction[23].

Immune-mediated hypersensitivity

Because liver undergoes continuous exposure to xenobiotics, it possesses a variety of local immune mechanisms to face these challenges. In the liver there are both innate and adaptive immune cells, including tissue macrophages (KC), natural Killer (NK) cells, and non-NK (NKT) cells, which account for nearly 50% of intrahepatic leukocytes[24]. KC produce various cytokines and other mediators, including prostanoids, nitric oxide and ROS that play roles in promoting and regulating hepatic inflammation. Furthermore, KC represent a major population of antigen-presenting cells (APCs) having an important role in the balance between the induction of immunity and tolerance within the liver. It has been demonstrated that freshly isolated liver NK cells spontaneously induce the cytotoxicity of various cell lines, whereas NKT cells are cytotoxic in the presence of interleukin (IL)-2[25]. This cytotoxicity is further enhanced by IL-12 and IL-18, which are produced by activated KC. Another function ascribed to NK and NKT cells is their ability to produce high levels of T helper (Th) 1 and Th2 cytokines upon stimulation[26]. NK cells have been shown to represent a major source of interferon (IFN)-γ in many types of liver disease[27]. NKT cells produce either IFN-γ or IL-4, or in some cases both cytokines, depending on the differentiation state of the cells and the stimuli[25]. It has also been demonstrated that IL-4 produced by NKT cells may be associated with the initiation and regulation of Th2 responses. Various mechanisms have been suggested to explain this tolerance[28], such as immune deviation, active suppression and apoptosis of activated T cells. Regarding immune deviation, it has been shown that Th2 cytokine production is preferentially maintained when adoptively transferred Th1 and Th2 cells are recovered from the liver. It has also been reported that liver sinusoidal endothelial cells (LSEC) are capable of selectively suppressing IFN-γ-producing Th1 cells while concurrently promoting the outgrowth of IL-4-expressing Th2 cells[29]. Good evidence suggests that hepatic dendritic cells are also important in the induction of tolerance, rather than the activation of T-cell responses. It has been further demonstrated that although LSEC are capable of presenting antigens to T cells, LSEC-activated $CD4^+$ or $CD8^+$ T cells fail to differentiate into Th1 cells or cytotoxic effector cells, respectively[30]. Most drugs are not chemically reactive but can be activated metabolically to reactive species which, after binding to cellular macromolecules, become immunogenic and can elicit an effective immune response. Immune-mediated mechanisms have been proposed for idiosyncratic reactions observed with sulfonamides, halothane and phenytoins. Presentation of drug-protein adducts by professional cells to Th lymphocytes, and/or a direct association between the drug and major histocompatibility complex (MHC) proteins of hepatocytes could be involved in the activation of the immune system. As a consequence of this, drug-directed antibodies and/or T-lymphocytes able to recognize drug-derived haptens arise which are responsible for the clinical manifestations of hepatitis. Drug-directed antibodies can be detected in sera of allergic patients by solid-phase immunoassays. Sensitized T-lymphocytes can be shown by hapten-induced cell proliferation experiments and by the early expression of CD69 antigen[31]. Despite the

possible detection of drug-specific antibodies, it is difficult to directly prove the pathogenic role of the adaptive immune system in DILI, partly because of the lack of animal models. A difficulty in developing animal models is the fact that the default response of the liver to antigens is immunological tolerance. This could also explain the relatively low occurrence of this type of DILI in human beings.

Inflammation

A recent paper emphasizes the imbalance between Th1 cells producing cytokines associated with a cell-mediated response and Th2 cells associated with an antibody response, leading to a shift in immune response to one that may participate in DILI during administration of certain drugs, especially in subjects with genetic polymorphisms in drug-metabolizing enzymes. In fact, several cases of DILI related to administration of drugs appear to be initiated or intensified by respiratory inflammation states, which stimulate sometimes dysregulated production of IFN-γ and/or other proinflammatory cytokines/growth factors. This ends up in down-regulation of various induced and constitutive isoforms of CYPs and other enzymes involved in the metabolism of several drugs, thus having an important impact on the alterations in bioactivation and detoxication processes. DILI may eventually be prevented by screening methods that can identify genetic polymorphisms of drug-metabolizing enzymes and gene polymorphisms or RNA-expression profiles of some proinflammatory cytokines before patients take any drug[32].

Apoptosis

Although CYP-generated reactive metabolites can cause hepatocyte apoptosis, the mechanism of this effect has only recently been elucidated. Male rat hepatocytes were incubated with skullcap diterpenoids. This treatment decreased cellular glutathione and protein thiols and increased cellular Ca^{2+}. This activated Ca^{2+}-dependent tissue transglutaminase formed a cross-linked protein scaffold, and also opened the mitochondrial permeability transition pore, causing outer mitochondrial membrane rupture, increased cytosolic cytochrome c, activation of procaspase 3, internucleosomal DNA fragmentation, and ultrastructural features of apoptosis. Cell death was increased by a CYP3A inducer (dexamethasone) increasing glutathione depletion. In contrast, cell death was prevented by decreasing CYP3A activity (with troleandomycin), preventing glutathione depletion (with cysteine), blocking Ca^{2+}-modulated events (with calmidazolium), preventing mitochondrial permeability transition (with cyclosporin A), or inhibiting caspase 3 (with acetyl-Asp-G u-Va-Asp-a dehyde). Both calmidazolium and cyclosporin A also prevented the increase in cytosolic cytochrome c and procaspase 3 activation[33].

Calcium homeostasis imbalance

When glutathione and other antioxidants are depleted, however, opportunities for lipid peroxidation are enhanced. Weakened cellular membranes allow sufficient leakage of Ca^{2+} into the cytosol to disrupt intracellular Ca^{2+} homeostasis. High Ca^{2+} levels in the cytosol activate Ca^{2+}-dependent proteases and phospholipases that further increase the breakdown of the membranes. Similarly, the increase in intracellular Ca^{2+} can activate endonucleases that can cause chromosomal damage and also contribute to cell death. Sustained cell regeneration and proliferation following cell death may increase the likelihood of unrepaired spontaneous, lipid peroxidation- or endonuclease-derived mutations that can lead to cancer.

FACTORS INFLUENCING PATIENT SUSCEPTIBILITY TO HEPATOXICITY

Determination of DILI also includes an individual susceptibility. This susceptibility is governed by genetic, pre-existing and environmental factors. Predisposing factors are generally thought to be important to somehow explain the unpredictability of the phenomena through which substances turn into hepatotoxins, and consist of ethnic and racial factors, CYP polymorphisms, concomitant liver diseases, age, nutritional status and diet, gender and pregnancy.

Ethnic and racial factors

These factors have important implications for susceptibility to acetaminophen hepatotoxicity following overdosage especially in a small subgroup showing extensive metabolic activation. An exemplary study indicates markedly reduced metabolic activation of acetaminophen in Africans. These ethnic differences in acetaminophen metabolism may be related to genetics even though environmental factors, including differences in diet and protein intake, should not be excluded. There were no ethnic differences in the sulphate conjugation of acetaminophen, but the mean fractional recovery of the glucuronide conjugate in Caucasians was less than in Africans[34].

Concomitant chronic liver diseases

In liver diseases, pharmacokinetics are generally impaired. Pathogenetic factors include alterations in intestinal absorption, plasma protein binding, hepatic extraction ratio, liver blood flow and portal-systemic shunting, biliary excretion, enterohepatic circulation, and renal clearance. The key point is, however, the reduction of functional hepatic mass that may have complex effects on drug clearance, particularly biotransformation. Net results for an individual drug are unpredictable and do not correlate well with the type of liver damage, its severity, or liver laboratory test results. Thus, no general rules are available for modifying drug dosage in patients with liver disease. Recently, NonAlcoholic Fatty Liver Disease (NAFLD) has been found to be a fertile soil for the development of hepatotoxicity. With NAFLD now linked to obesity and metabolic syndrome, the impact of this observation should not be overlooked[35].

Oxidative stress has been detected in patients affected by alcohol abuse, hepatitis C virus (HCV) infections, iron overload and chronic cholestasis[36]. Alcohol-induced liver

disease (ALD) has been associated with the synergistic induction of oxidative stress by alcohol metabolites, iron accumulation and antioxidant depletion[37].

HCV infection may generate oxidative stress by chronic inflammation and by disruption of glutathione efflux. Therefore, oxidative stress is not only a consequence of chronic liver injury but it also contributes to fibrogenesis and it appears as a key player in the pathogenesis of hepatic diseases. Vitamin E could prevent the decrease in O_2 uptake[38].

But what is the role of stress in determining hepatotoxicity? Its regulation of several enzymatic systems which are involved in the biotransformation of xenobiotics in the liver was recently investigated in a study using restraint stress as a stress model in animals. The results demonstrated that stress suppressed total basal CYP content of one third of animals and basal ethoxyresorufin 7-dealkylase activity. On the other hand, restraint stress increased total CYP content in 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene-treated mice, while slightly suppressing PROD activity. In addition, CYP2E1 dependent p-nitrophenol hydroxylation was suppressed by stress in the same animals and cytosolic aldehyde dehydrogenases were not affected. Although stress had no effect on basal CYP2A5 activity, the inducibility of this hepatic activity increased 2-fold after stress exposure. In addition, a slight suppression in liver glutathione content was found. Northern blot analysis revealed that restraint stress had a relatively suppressive effect on control CYP1A2 expression in the liver. In conclusion, stress was found to significantly interfere with the expression processes of some CYP450s[39].

Age

Increased risk of DILI may result from age-related changes in pharmacokinetics or pharmacodynamics. Overall hepatic metabolism of many drugs through the CYP enzyme system decreases with aging. For drugs with decreased hepatic metabolism, clearance typically decreases by 30%-40%. Theoretically, maintenance drug doses should be decreased by this percentage; however, the rate of drug metabolism varies greatly from person to person, and individual titration is required. Risk of any adverse effect and obviously of DILI increases exponentially with the number of drugs used, partly because multiple drug therapy reflects the presence of many diseases and increases risk of drug-disease and drug-drug interactions.

Nutritional status and diet

Increasing evidence implicates dietary factors in the progression of diseases, including certain cancers, diabetes and obesity. Diet also regulates the expression and function of CYP genes which impacts on drug elimination and may also significantly affect disease pathogenesis. Upregulation of CYPs 2E1 and 3A4 occurs after feeding of experimental diets that are high in fats or carbohydrates; these diets also promote hepatic lipid infiltration, which is a component of the metabolic syndrome that characterizes obesity. Increased availability of lipid substrates for CYPs can enhance free radical production and exacerbate tissue injury. Similar processes may also occur in other models of experimental disease states that exhibit a component of altered nutrient utilization. Food-derived chemicals, including constituents of cruciferous vegetables and fruits, modulate CYP expression and the expression of genes that encode cytoprotective phase Ⅱ enzymes. Certain dietary indoles and flavonoids activate CYP1A expression either by direct ligand interaction with the aryl hydrocarbon receptor (AhR) or by augmenting the interaction of the AhR with xenobiotic response elements in CYP1A1 and other target genes. Other dietary chemicals, including methylenedioxyphenyl (MDP) compounds and isothiocyanates also modulate CYP gene expression. Apart from altered CYP regulation, a number of dietary agents also inhibit CYP enzyme activity, leading to pharmacokinetic interactions with coadministered drugs. A well described example is that of grapefruit juice, which contains psoralens and possibly other chemicals that inactivate intestinal CYP3A4. Decreased presystemic oxidation by this CYP increases the systemic bioavailability of drug substrates and the likelihood of drug toxicity. Dietary interactions may complicate drug therapy but inhibition of certain CYP reactions may also protect the individual against toxic metabolites and free radicals generated by CYPs. Chemicals in teas and cruciferous vegetables may also inhibit human CYP enzymes that have been implicated in the bioactivation of chemical carcinogens. Thus, food constituents modulate CYP expression and function by a range of mechanisms, with the potential for both deleterious and beneficial outcomes[40].

As previously emphasised, obesity is considered an important risk factor for DILI. Paradoxically, earlier studies have shown highly exaggerated mechanism-based liver injury by thioacetamide (TA) in rats following moderate diet restriction (DR). The objective of this significant study was to investigate the mechanism of higher liver injury of TA in DR rats. When male rats were maintained on DR (35% of ad libitum diet for 21 d), the total hepatic CYP was increased 2-fold along and there was a 4.6-fold increase in CYP2E1 protein, which corresponded with a 3-fold increase in CYP2E1 activity as measured by chlorzoxazone hydroxylation. To further test the involvement of CYP2E1, 24 and 18 h after pretreatment with pyridine (PYR) and isoniazid (INZ), specific inducers of CYP2E1, rats received a single administration of 50 mg of TA/kg. TA liver injury was > 2.5- and > 3-fold higher at 24 h in PYR + TA and INZ + TA groups, respectively, compared with the rats receiving TA alone. Pyridine pretreatment resulted in significantly increased total CYP content accompanied by a 2.2-fold increase in CYP2E1 protein and 2-fold increase in enzyme activity concordant with increased liver injury of TA, suggesting mechanism-based bioactivation of TA by CYP2E1. Hepatic injury of TA in DR rats pretreated with diallyl sulfide (DAS), a well known irreversible *in vivo* inhibitor of CYP2E1, was significantly decreased (60%) at 24 h. Carbon tetrachloride (CCl_4), a known substrate of CYP2E1, caused less liver injury and greater animal survival, confirming inhibition of CYP2E1 by DAS pretreatment[41].

Gender

Some authors clarified this issue, studying 50 patients diagnosed with active tuberculosis infection with normal pretreatment liver laboratory tests; they were monitored clinically as well as biochemically in a prospective cohort analysis. Antitubercular drug-induced hepatotoxicity was found more often in females (OR 4.2). Younger patients were also at a higher risk (OR 2.75). Nutritional status, assessed by body mass index and serum albumin level, was the next most common predisposing factor[42].

PHENOTYPING AND GENOTYPING STUDIES

The genetic polymorphisms of human drug metabolizing enzymes have been firmly established. Based on the metabolic handling of certain probe drugs, the population can be divided into two phenotypes: the rapid acetylator/extensive metabolizer (EM) and slow acetylator/poor metabolizer (PM). For some authors, a quadrimodal behaviour is possible, that is, poor, intermediate, extensive and ultrarapid.

These polymorphisms have provided useful tools to study the relationship between genetically determined differences in the activity of drug metabolizing enzymes and the risk for adverse drug reactions and certain types of chemically-induced diseases.

With regard to the susceptibility of the two phenotypes, DILI can be anticipated for the following scenarios: (1) the drug toxicity is caused by the parent compound and the elimination of the drug proceeds exclusively *via* the polymorphic enzyme, there being no alternate pathways of biotransformation available. Thus the poor metabolizer phenotype will be more prone to such a type of toxicity since, at the same level of exposure, this phenotype will accumulate the drug as a result of impaired metabolism; (2) the polymorphic pathway is a major route of detoxification, so impairment of this pathway shifts the metabolism to an alternate pathway *via* which a reactive intermediate is formed. In such a situation the PM phenotype constitutes a major risk factor for toxicity (i.e. INZ hepatotoxicity); the toxicity is mediated by a reactive intermediate generated by a polymorphic enzyme. Hence EMs are at a much higher risk than PMs of developing toxicity or cancer (for example, smokers)[43].

A further problem with PMs that the same dose of drug could yield a sustained plasma concentration. Poor metabolism is especially problematic with drugs that have a narrow therapeutic index like debrisoquine, phenformin or captopril. It has been estimated that psychiatric patients with CYP2D6 deficiency encounter more adverse drug incidents than those who are EMs[44].

With estimates of the percentage of pharmaceuticals that are subject to metabolism by the CYP in excess of 80%, the relative activities of these enzymes in various subpopulations and even in individual patients can have important ramifications in matters ranging from dose selection to prediction of toxicity to suitability of a new chemical entity for continued drug development.

As previously emphasized, CYP1A2, 2C9, 2C19, 2D6 and 3A are the major isoforms responsible for the metabolism of more than 90% of marketed drugs. Polymorphism of drug metabolism represents an important source of interindividual and interethnic variation in drug response.

CYP2D6, CYP2C9 and CYP2C19 are three polymorphic CYP enzymes. The EM phenotype occurs when there is at least one wild type allele at the relevant gene locus. The PM phenotype occurs when both alleles of either CYP2D6 or CYP2C19 carry inactivating mutations and give rise to synthesis of enzyme with impaired activity or no synthesis of enzyme at all.

Phenotyping is often based on high-performance liquid chromatography (HPLC) such as the determination of the dextromethorphan/total dextrorphan molar ratios as metabolic ratios (MRs) in plasma samples collected at 3 h after oral administration of 30 mg dextromethorphan hydrobromide. In this situation PMs and extensive EMs can be identified distinctly. To determine the real-time activity of the CYP isozymes, specific probe drugs can be employed. Recently, the use of multiple probe drugs, that is, a 'cocktail' approach, has become popular in pharmacogenetic studies as this provides a high-throughput approach in evaluating CYP isozyme activities. A number of cocktails (from five to six drugs) have been described in the literature.

These include the Pittsburgh cocktail, GW cocktail, Cooperstown cocktail and Karolinska cocktail. So far most of the analytical methods for these cocktails usually require a separate HPLC, gas chromatography (GC) or liquid chromatography/mass spectrometry (LC/MS) technique for each probe drug and its metabolite.

Recently, a fast gradient LC/MS method for the simultaneous determination of CYP substrates and metabolites in the GW cocktail was reported[45]. However, this cocktail has several practical limitations. First of all, the use of diclofenac as a CYP2C9 marker is undesirable due to its variable absorption in humans. Secondly, the use of mephenytoin is inconvenient as this drug is no longer commercially available in many parts of the world; besides, its sedative side effect is prominent, especially in PMs. Thirdly, chlorzoxazone, a probe drug in the cocktail, can significantly inhibit the CYP3A-mediated first-pass metabolism of midazolam in the gut and its use for the present purpose is not recommended.

A rapid LC/tandem MS method has been developed for the determination of six CYP probe substrate metabolites including acetaminophen for CYP1A2, 4-hydroxytolbutamide for CYP2C9, 5-hydroxyomeprazole for CYP2C19, dextrorphan for CYP2D6, 6-hydroxychlorzoxazone for CYP2E1 and dehydronifedipine for CYP3A4[46].

What is the clinical importance of studying the CYP polymorphisms? It has been shown that the cholesterol-lowering effect as well as the efficacy and tolerability of simvastatin is influenced by CYP2D6 genetic polymorphism[47]. Because the different HMG-CoA reductase in-

hibitors differ with respect to the degree of metabolism by the different CYP enzymes, genotyping may help to select the appropriate HMG-CoA reductase inhibitor and the optimal dosage during the start of the treatment and will allow for more efficient individual therapy, also taking in account the eventual DILI. To clarify this point, CYP polymorphisms of fluvastatin were studied.

More than the hepatotoxicity, the pharmacokinetics of both enantiomers of fluvastatin depended on the CYP2C9 genotype with a 3-fold mean difference in the active enantiomer and even greater differences in the inactive enantiomer. Differences in plasma concentrations were not reflected in cholesterol lowering after 14 d of fluvastatin intake in healthy volunteers[48]. In fact, the authors did not find any evidence to support CYP2C9 and CYP2C19 genetic polymorphisms as predictable potential risk factors for DILI[49].

PREDICTING VARIABILITY IN PHARMACOKINETICS

Obviously, it is impossible to genotype all individuals due to the cost, even though for some drugs it should be taken into serious consideration because a single serious incident (hepatic failure) could lead to an excessive health care involvement (liver transplantation). Several examples have been reported.

CYP2C19 is highly polymorphic, with variations in both the expression of mRNA and enzyme, plus actual differences in the protein coding region that give rise to differing rates of catalysis. As with most polymorphisms, there appear to be differences in expression in different ethnic groups. For example, the frequency of PMs among Asians is nearly 20% of Caucasians[50]. The proton pump inhibitor omeprazole and related ulcer drugs are oxidized by CYP2C19, and PMs show a better response to these drugs[51].

Not all drug interactions are genetically determined. In some cases, an inhibitor can block metabolism of a drug and produce the same effect as would poor metabolism. In retrospect, every marketed drug is a relatively successful drug in terms of the limited problems associated with widespread use, the deaths of some individuals notwithstanding-as in the case of "ultra" rapid metabolizers-or, more commonly, arising from enzyme induction. A classic example involves CYP3A4 and 17α-ethynylestradiol, the estrogenic component of oral contraceptives. Similarly, hyperforin, a potent CYP inducer found in the herbal medicine St. John's wort, greatly increases the expression of CYPs that metabolize drugs used for AIDS treatment and organ transplantation. Cases for enhanced drug toxicity due to elevated levels of CYPs are probably less clear; however, CYP3A4 converts the antidiabetic drug troglitazone into toxic products, although the mechanism of toxicity is still unclear. Troglitazone has since been removed from the market. In any event, the drug development process now incorporates a variety of *in vitro* studies designed to predict bioavailability, inhibition of CYP reaction, and the effects of any induction prior to consideration of clinical trials. Conclusively, genotyping can provide useful information about the expected behavior of a drug.

HAS CYP A ROLE IN THE "DIRECT" HEPATOTOXICITY?

CCl_4 is a well-known model compound for producing chemical hepatic injury. CYP is an important monooxygenase in biology. Recent research investigated CYP protein expression in the *in vivo* hepatotoxicity of rats induced by CCl_4. In this experiment, CCl_4 was administered to male rats, and their livers at 24 h post-dosing were studied using proteomic analysis. Blood biochemistry and histopathology were examined to identify specific changes. At the same time, a novel acetylation stable isotopic labeling method coupled with LTQ-FTICR MS was applied to disclose the changes in CYP expression amounts. The quantitative proteomics method demonstrated its correlation coefficient was 0.9998 in a 100-fold dynamic range and the average ratio of the labeled peptides was 1.04, which was very close to the theoretical ratio of 1.00 and the standard deviation (SD) of 0.21. With this approach, 17 CYP proteins were identified and quantified with high confidence. Among them, the expression amounts of 2C11, 3A2, and 2 E1 were down-regulated, while those of 2C6, 2B2, and 2B1 were up-regulated[52].

AN EXAMPLE OF DAMAGE MECHANISMS

CCl_4 continues to provide an important service today as a model substance to elucidate the mechanisms of action of hepatotoxic effects such as fatty degeneration, fibrosis, hepatocellular death, and carcinogenicity. CCl_4 is activated by CYP2E1, CYP2B1 or CYP2B2, and possibly CYP3A, to form the trichloromethyl radical, CCl_3*. This radical can bind to cellular molecules (protein, lipid, nucleic acids), impairing crucial cellular processes such as lipid metabolism, leading to fatty degeneration (hepatic steatosis). Adduct formation between CCl_3* and DNA is thought to possibly induce hepatic cancer. This radical can also react with O_2 to form the trichloromethylperoxy radical CCl_3OO*, a highly reactive species, also called ROS. CCl_3OO* initiates the chain reaction of lipid peroxidation, which attacks and destroys polyunsaturated fatty acids, in particular those associated with phospholipids. This affects the permeabilities of mitochondrial, endoplasmic reticulum, and plasma membranes, resulting in the loss of cellular Ca^{2+} sequestration and homeostasis, which can contribute heavily to subsequent cell damage. Among the degradation products of fatty acids are reactive aldehydes, especially 4-hydroxynonenal, which bind easily to functional groups of proteins and inhibit important enzyme activities. CCl_4 intoxication also leads to hypomethylation of cellular components; in the case of RNA the outcome is thought to be inhibition of protein synthesis, and in the

case of phospholipids it plays a role in the inhibition of lipoprotein secretion. None of these processes per se is considered the ultimate cause of CCl_4-induced cell death; it is by cooperation that they achieve a fatal outcome, provided the toxicant acts in a high single dose, or over longer periods of time at low doses. At the molecular level CCl_4 activates tumor necrosis factor (TNF)-α, nitric oxide, and transforming growth factor (TGF)-α and -β in the cell, processes that appear to direct the cell primarily toward (self-)destruction or fibrosis. TNF-α pushes toward apoptosis, whereas TGF-β appears to direct toward fibrosis. IL-6, although induced by TNF-α, has a clearly antiapoptotic effect, and IL-10 also counteracts TNF-α action. Thus, both interleukins have the potential to initiate recovery of the CCl_4-damaged hepatocyte. Several of the above-mentioned toxicological processes can be specifically interrupted with the use of antioxidants and mitogens, respectively, by restoring cellular methylation, or by preserving calcium sequestration. Chemicals that induce CYPs that metabolize CCl_4, or delay tissue regeneration when co-administered with CCl_4 will potentiate its toxicity thoroughly, while appropriate CYP inhibitors will alleviate much of the toxicity. O_2 partial pressure can also direct the course of CCl_4 hepatotoxicity. Pressures between 5 and 35 mmHg favor lipid peroxidation, whereas the absence of O_2, as well as a partial pressure above 100 mmHg, both prevent lipid peroxidation entirely. Consequently, the location of CCl_4-induced damage mirrors the O_2 gradient across the liver lobule. Mixed halogenated methanes and ethanes, found as so-called disinfectant by-products at low concentrations in drinking water, elicit symptoms of toxicity very similar to CCl_4, including carcinogenicity[23].

TOTAL DOSE OF DRUG

Among drugs that are feared of inducing hepatoxicity, mainly when taken for a very long period, statins are largely under-dosed, but they do in rare cases cause significant liver injury whereas antiretroviral therapy is associated with hepatotoxicity in 10% of treated patients[16].

In a previous study, liver morphology was examined in 41 patients with vitamin A hepatoxicity. Cirrhosis was found in 17, mild chronic hepatitis in 10, noncirrhotic portal hypertension in 5, and "increased storage" alone in nine cases. During a mean follow-up period of 4.6 years, six patients died of causes related to the liver disease. A precise appraisal of drug consumption was obtained in 29 cases. Among them the total cumulative intake was highest in patients with cirrhosis ($423 \pm 103 \times 10^6$ IU) and significantly lower in those with noncirrhotic liver disease (88.5 ± 41; $P < 0.02$). The smallest continuous daily consumption leading to cirrhosis was 25 000 IU during 6 years, whereas higher daily doses (⩾ 100 000 IU) taken over 2.5 years resulted in similar histological lesions. It was concluded that prolonged and continuous consumption of doses in the low "therapeutic" range can result in life-threatening liver damage[53].

Recently, an interesting paper, reporting data from both USA and Sweden, showed a clear relationship between daily doses of oral prescription medications and idiosyncratic DILI, particularly as regards daily doses > 50 mg/d[54].

INDUCTION AND INHIBITION OF CYP ACTIVITY

The possible pharmacokinetic consequences of enzyme induction depend on the localization of the enzyme. They include decreased or absent bioavailability for orally administered drugs, increased hepatic clearance or accelerated formation of reactive metabolites, which is usually related to local toxicity. The toxicological consequences of enzyme induction in humans are fortunately rare, and appear to be mainly limited to hepatoxicity in ethanol-type induction[55].

Diclofenac sodium (DF-Na) is an NSAID used in various aspects of inflammatory disease. The effects of phenobarbital (PB) on metabolism and toxicity of DF-Na *in vitro* and the potential mechanism of DF-Na induced hepatotoxicity have been examined. The decline of CYP 3A was partially reversed by CYP inducer PB, and the maximum induction of CYP 3A was 2.2-fold over control after continuous exposure of hepatocytes to 2 mmol/L PB for 48 h. These findings suggest that the hepatotoxicity and metabolism of DF-Na in rat hepatocytes are increased when hepatic CYP 3A activity is increased[56]. Itraconazole and fluconazole, two antifungal drugs with potent inhibitory effect on CYP, induce hepatotoxicity clinically, but the mechanism underlying the hepatotoxicity is unknown. Pretreatment with SKF 525A, an inhibitor of CYPs, induced more severe hepatotoxicity with both itraconazole and fluconazole *in vivo*[57].

THE IMPACT OF DILI ON DRUG DEVELOPMENT

The inability to predict if a metabolically bioactivated compound will cause toxicity in later stages of drug development or post-marketing is of great importance. One approach for improving the predictive success of compound toxicity could be to compare the gene expression profile in preclinical models dosed with novel compounds to a gene expression database generated from compounds with known toxicity.

A current study[58] in mice utilized a known hepatotoxic compound N-methylformamide and its two analogs labeled with deuterium at different positions to block metabolic oxidation at the formyl [d (1)] and methyl [d (3)] moieties. The data set generated from the different groups of animals enabled authors to determine which gene expression changes were attributed to the bioactivating pathway. The metabolic pathway leading to the production of reactive methyl isocyanate resulted in distinct expression patterns that correlated with histopathologic findings. There was a clear correlation between the expression of certain genes involved in the cell cycle/apoptosis and inflammatory pathways and

the presence of reactive metabolite. These genes may serve as potential genomic biomarkers of hepatotoxicity induced by soft-electrophile-producing compounds. However, the robustness of these potential genomic biomarkers will need to be validated before being adopted into the drug discovery screening process.

AN OPINION ABOUT WIDELY USED DRUGS

Although liver injury has been associated with the "statins", the frequency of such toxicity is lower than that of the control population and the value of biochemical monitoring remains unproved[59]. Clinicians may be concerned about prescribing statins to patients with chronic liver disease, but there is little evidence to suggest that DILI from statins is increased in these patients. Thus, we should prescribe statins for the same indications in patients with chronic liver disease as in patients without, but with closer monitoring[60].

HERBAL REMEDIES AND OTHER DIETARY SUPPLEMENTS

A dietary supplement is defined as any product in pill or liquid form containing a herb, vitamin, amino acid or mineral that complements the normal diet. Indeed, every agency regulates dietary supplements differently from drugs, i.e. only ensuring quality control and good manufacturing processes but not standardization of the active ingredients. Dietary supplements are commonly used primarily because they are widely available and can be bought without consulting a physisician. However, a few supplements are now proven to be safe and useful complements to standard drugs. The supplement may have unlisted ingredients, which may be inert or harmful, or it may contain variable amounts of active ingredients, especially when whole herbs are ground or made into extracts. Most herbal products are mixtures of several substances, and which ingredients are active is not always known. Additional areas of concern include stability of supplements (especially herbal products) once manufactured, use of dietary supplements instead of conventional drugs, toxicity in children and the elderly, and interactions between supplements and drugs. Patients may not think to disclose or may wish to conceal their use of dietary supplements. For this reason, the patient's history should periodically include explicit questions about past and recent consumption of dietary supplements. Recently the use of herbal preparations as remedies for various medical conditions has been increasing rapidly. In one study, 38.9% of patients with chronic liver disease were found to use some sort of herbal preparation[61].

Efficacy and safety of medicinal plants naturally represent the object of interest for pharmacologists, and it is surely this aspect which gives the most important information on herbal medicine use[62].

Many plants have been studied and results published showing variable degrees of efficacy. Toxicity aspects of some of the most frequently used plants are now well known[63].

Among others, a recent report emphasizes the potentially severe hepatotoxicity of Kava which has recently led to the retraction of Kava-containing drugs by the pharmacovigilance authorities in Germany[64]. Authors reported two cases of acute liver injury along with the intake of greater celandine (*Chelidonium majus*), a well-known herbal remedy frequently used for irritable bowel syndrome. All other possible causes of acute liver damage were excluded in both patients. In one patient, cholestatic hepatitis recurred rapidly after involuntary re-exposure. Both patients fully recovered after the withdrawal of greater celandine. The two cases add to the existing database about the potential hepatotoxicity of drugs containing greater celandine and raise the question whether the approval of this drug should be re-evaluated in the light of a lack of evidence for a therapeutic benefit[65].

SOME CONSIDERATIONS ON CAUSALITY ASSESSMENT

Many methods have been proposed to assess the individual causality between a drug treatment and the occurrence of adverse drug events (ADRs), including hepatoxicity. Briefly, these methods may be classified into the following approaches, i.e. expert judgement, probabilistic methods and algorithms.

In expert judgement or global introspection (GI), an expert expresses a judgement about possible drug causation after having taken into account all the available and relevant information on the considered case.

Theoretically, it is possible to apply pre-existing Causality Assessment Methods (CAMs) to the assessment of causality in cases with diagnostic difficulties.

We have historical scales such as Naranjo probability scale[66], Danan's international consensus criteria[67], Maria's and Victorino's scales[68] or Beers criteria for ADRs[69] to make such events predictable and often preventable. Still, on the basis of a global score, four categories of preventability of ADRs ("preventable", "potentially preventable", "unclassable", "not preventable") were proposed by other researchers[70].

Standards are lacking for validation of drug CAMs. An original model has been proposed using a positive rechallenge as an external standard[71]. The GI approach suffers from marked subjectivity leading to poor reproducibility and intra- and inter-rater disagreements. A study confirms that experts express marked disagreements when assessing drug causality independently. The agreement rate was lower for intermediate levels of causality, especially when strong evidence was lacking for confirming or ruling out drug causality[72]. Probabilistic methods are usually regarded as the most rigorous[73]. The probabilistic approach is based on the Bayes theorem and makes it possible to directly assess the odds of drug causation. However, these methods are rather troublesome to routinely use because information for

assessing the probability of drug causation is rarely available. Unlike the Bayesian approach, algorithms have appealing simplicity and are much more widely used for the operational assessment of ADRs. The main reason for their use is to increase inter- and intra-rater agreement. The overall observed agreement between algorithm and GI was moderate although poorly different from chance, confounding variables being a shortcoming of algorithms ability in assessing causality[74].

Drawbacks of animal models

The doubtful assumption that animal models are reasonably predictive of human outcomes has provided the basis for their widespread use in toxicity testing and in biomedical research aimed at developing cures for human diseases. To investigate the validity of this assumption, comprehensive bibliographic databases were searched for published systematic reviews of the human clinical or toxicological utility of animal experiments. In 20 reviews in which clinical utility was examined, the authors concluded that animal models were either significantly useful in contributing to the development of clinical interventions, or were substantially consistent with clinical outcomes, in only two cases, one of which was contentious. These reviews failed to clearly demonstrate utility in predicting human toxicological outcomes; consequently, animal data may not generally be assumed to be substantially useful for these purposes. Possible causes include interspecies differences, the distortion of outcomes arising from experimental environments and protocols, and the poor methodological quality of many animal experiments. What is more, very few reviews existed in which the majority of animal experiments were of good methodological quality. The poor human clinical and toxicological utility of most animal models for which data exists, in conjunction with economic costs, justify a perplexity on animal models[75].

In numerous cases, researchers are simply not aware of the limitations of the animal experiment as such. For example, many animal experiments are dramatically "under-powered", that is, carried out with groups that are too small to allow conclusions to be drawn from the outcome. This stands in marked contrast to *in vitro* experiments where replicate experiments usually represent no major problem. Since *in vitro* models are generally more prone to artefacts due to the numerous variables, for example, of cell culture, the key requirement for their application is their validation and quality control. Sadly, many methods, even if published in the scientific literature, are little standardised and reproducible. Due to limitations in space, many scientific journals cannot publish detailed methodological descriptions. However, nowadays a supplementary central deposit of methods could easily be linked to the respective article[76].

ETHICAL AND LEGAL PROBLEMS ABOUT DRUG-INDUCED LIVER INJURY

Patients should be especially cautious about combining multiple drugs, and tell their doctor about any drugs or other substances they are taking, including prescription and over-the-counter medications, recreational drugs, herbal remedies, and nutritional supplements. Health care professionals are encouraged to report all ADRs, mainly hepatoxicity, and to pay much more attention in prescribing and administering drugs.

Pharmacovigilance is a key step in discovering DILI. But, it is also concerned with pharmacological, pathological, epidemiological and legal respects, other ADRs and interactions as well as problems relating to ineffectiveness, inappropriate use, dependence or poisoning. Physicians should always think of this ADR in the absence of other clear hepatic disease.

LIVER DISEASES POTENTIALLY CAUSED BY DRUGS

Acute hepatitis

Dose-dependent: Acetaminophen[77,78], salicylates[79], (high doses i.e. > 2 g/d).

Dose-independent: Acebutolol[80], allopurinol[81], carbamazepine[82], cimetidine[83], dantrolene[84], diclofenac[79], ethambutol[85], ethionamide[86], enflurane[87], phenelzine[88], phenindione[89], phenobarbital[90], phenytoin[91], phenylbutazone[79], halothane[92], ibuprofen[79], indomethacin[79], isoniazid[85], ketoconazole[93], labetalol[94], maprotiline[95], metoprolol[96], mianserin[97], naproxen[79], para-aminosalicylic acid[98], piroxicam[79], pyrazinamide[85], quinidine[99], penicillins[100], ranitidine[101], sulfonamides[102], sulindac[79], tricyclic antidepressants[103], trimethoprim-sulfamethoxazole[104], valproic acid[105], verapamil[106].

Acute fatty liver

Adrenocortical steroids[107], phenytoin[108], salicylates[79].

Fatty liver

Amiodarone[109], asparaginase[110], ibuprofen[79], indometacin[79], ketoconazole[111], methyldopa[112], naproxen[79], nifedipine[113], acetaminophen[77], perhexeline[114], rifampicin[85], sulindac[79], tetracyclin[115], valproic acid[116], zidovulin[117].

Cholestatic syndrome

Amoxicillin/clavulanate[118], azathioprine[119], captopril[120], carbamazepine[121], carbimazole[122], cephalosporins[123], chlordiazepoxide[124], chlorpropamide[125], cloxacillin[126], cyclosporine[127], danazol[128], disopyramide[129], enalapril[130], erythromycin[131], flecainide, flucoxacillin[132], flurazepam[133], flutamide[134], gold[135], griseofulvin[136], glyburide[137], imipramine[138], haloperidol[139], ketoconazole[140], megestrol[141], mercaptopurine[119], methimazole[142], methyltestosterone[143], nifedipine[144], nitrofurantoin[145], norethandrolone[146], nonsteroidal anti-inflammatory drugs[79], oral contraceptives[147], phenothiazines[148], phenytoin[149], penicillamine[150], propoxyphene[151], sulfonamides[152], tamoxifen[153], thiabendazole[154], tolbutamide[155], tricyclic antidepressants[156], troleandomycin[157], verapamil[158].

Liver granulomas

Allopurinol[159], aspirin[79], carbamazepine[160], chlorpromazine[161], diltiazem[162], gold[163], hydralazine[164], nitrofurantoin[165], penicillin[166], phenylbutazone[79], phenytoin[167], pyrazinamide[168], quinidine[169], sulfasalazine[170].

Chronic liver disease

Acetaminophen (in chronic use or at high doses)[79], dantrolene[171], isoniazid[172], methyldopa[173], phenytoin[174].

Liver cirrhosis/fibrosis

Methotrexate[175], nitrofurantoin[176], terbinafine[177].

Liver tumors

Anabolic steroids[178], danazol[179], oral contraceptives[178], testosterone[178], thorotrast[180].

Vascular reactions

Anabolic steroids[181], azathioprine[182], cyclophosphamide/cyclophosphamide combination[183], dacarbazine[184], oral contraceptives[185], thioquanine[186], vincristine[187].

Fulminant hepatitis and hepatic failure

Lamotrigine[188], nimesulide[189], carbamzepine and levetiracetam[190], isoniazid[191], clarithromycin[192], ecstasy[193].

LIVER DISEASES EVENTUALLY CAUSED BY DIETARY SUPPLEMENT (MAINLY IN OBESE PATIENTS)

Germander (Teucrium chamaedrys) extracts, widely used in Europe in the last decades as a weight loss agent, cause DILI probably mediated by furano neoclerodane diterpenoids[194]. Chaparral (creosote bush, greasewood, or Larrea tridentata) is a desert shrub traditionally used by Native Americans for treatment of several ailments. More recently, preparations of chaparral leaves have been marketed for use as weight loss agents. Reports of chaparral hepatotoxicity were first seen in 1992. The mechanism of chaparral toxicity is unclear but may involve its active ingredient, nordihydroguaiaretic acid[195]. Kava (kava kava, awa, or kew), derived from the dried root and rhizome of Piper methysticum, has recently been marketed as an anxiolytic and mood enhancer. Recent series from Europe have described more than 30 cases of kava-associated hepatic injury, including five cases leading to OLT. The mechanism of hepatic injury appears to be immune-mediated, with CYP2D6 deficiency perhaps being a risk factor[196]. *Ma huang* (from Ephedra sinica and other Ephedra species) is a traditional Chinese extract used for treatment of asthma, nasal congestion, and fever. Recent Western marketing has focused on the stimulatory effects of *Ma huang*, which contains 0.15%-2% of ephedrine-like alkaloids by weight. Although most adverse effects of *Ma huang* are cardiovascular or neurological (e.g. hypertension, stroke, myocardial infarction, seizures, and psychosis), 4% of reports mentioned acute hepatitis. *Ma huang* contains phytochemicals which are thought to modify its toxic activity[197]. In addition to the above supplements, liver injury has been attributed to other botanical agents. The pyrrolizidine alkaloids found in comfrey leaves and Heliotropium, Senecio, and Crotalaria species are known to cause veno-occlusive disease of the liver *via* a toxic effect[198]. Mixtures of valerian and skullcap (Valeriana officinalis and Scutellaria lateriflora) have induced hepatitis *via* alkylating agents. LipoKinetix, was marketed as a dietary supplement for weight loss. Following reports of seven cases of severe hepatotoxicity associated with its use, the FDA moved to remove it from the market in November 2001. Hepatic injury appears to be due to an idiosyncratic reaction, perhaps related to phenylpropanolamine[199]. Among other weight loss agents, usnic acid should be suspected in cases of severe hepatotoxicity[200].

For further details about the topics of this report, we advise the readers to consider the review by Bleibel *et al*[201] and/or to connect to: http://www.fda.gov/cder/guidance/7507dft.htm.

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EDITORIAL

Consequences of dysthyroidism on the digestive tract and viscera

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Abstract

Thyroid hormones define basal metabolism throughout the body, particularly in the intestine and viscera. Gastrointestinal manifestations of dysthyroidism are numerous and involve all portions of the tract. Thyroid hormone action on motility has been widely studied, but more complex pathophysiologic mechanisms have been indicated by some studies although these are not fully understood. Both thyroid hormone excess and deficiency can have similar digestive manifestations, such as diarrhea, although the mechanism is different in each situation. The liver is the most affected organ in both hypo- and hyperthyroidism. Specific digestive diseases may be associated with autoimmune thyroid processes, such as Hashimoto's thyroiditis and Grave's disease. Among them, celiac sprue and primary biliary cirrhosis are the most frequent although a clear common mechanism has never been proven. Overall, thyroid-related digestive manifestations were described decades ago but studies are still needed in order to confirm old concepts or elucidate undiscovered mechanisms. All practitioners must be aware of digestive symptoms due to dysthyroidism in order to avoid misdiagnosis of rare but potentially lethal situations.

Key words: Hypothyroidism; Hyperthyroidism; Gastrointestinal motility; Intestine; Liver; Viscera

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INTRODUCTION

Thyroid hormones act on almost all organs throughout the body and regulate the basal metabolism of the organism[1]. The gut and viscera are not spared, and disturbances in thyroid function have numerous gastrointestinal manifestations, the true incidence of which is unknown[2]. Digestive symptoms or signs may also reveal clues to thyroid disease and, when ignored or underestimated, diagnosis may be delayed and serious consequences may occur[3-5]. Additionally, patients with dysthyroidism are at an increased risk of developing specific pathologies in the digestive system, whether due to thyroid hormone disturbances or associated with a particular thyroid disease[6-17].

Thyroid interactions with the gastrointestinal system have been widely reported but the literature lacks an exhaustive report on different consequences of dysthyroidism. Gastrointestinal motor dysfunction has been widely accepted as the main cause of symptoms but many complex phenomena have not yet been completely elucidated[4,11,18-21]. This review aims to gather up-to-date knowledge about the effects of dysthyroidism on the gut and viscera.

HYPERTHYROIDISM

As thyroid hormones act on almost all organs within the gastrointestinal tract (gut and viscera), hyperthyroidism induces several symptoms and signs, and causes different biologic and metabolic derangements. Digestive symptoms may represent the only manifestations of hyperthyroidism. A lack of cardinal features of the disease and the presence of persistent abdominal pain, intractable vomiting, weight loss and altered bowel habits are designated as apathetic hyperthyroidism[22].

Esophagus and stomach

Dysphagia is a rare manifestation of hyperthyroidism and can have an acute or chronic pattern[3]. It may be related to direct compression from goiter or to altered neurohormonal regulation[23,24]. Excess thyroid hormone may cause myopathy which involves striated muscles of the pharynx and the upper third of the esophagus[23]. Subsequently, the oropharyngeal phase of deglutition is predominantly impaired and patients are predisposed to nasal regurgitation and aspiration pneumonia. Correction of the endocrine disorder is believed to reverse dysphagia[3,23,24].

In the esophagus, thyroid hormone excess increases the propagation velocity of contractions[25]. Thyrotoxic patients may frequently complain of chronic dyspeptic symptoms such as epigastric pain, fullness and eructation[2]. Tachygastria has been incriminated in upper gastrointestinal symptoms but the true mechanism is not yet fully understood[19,20]. Vomiting is rarely intractable and may involve neurohormonal mediators along with direct action[26]. Studies have yielded variable, even contradictory results concerning gastric emptying in thyrotoxicosis[18,19,27,28]. A significant increase in the dominant electrical frequency and dysrhythmia was shown through a myoelectrical activity study[19,20] but lack of correlation between electrogastrography (EGG) findings and gastric emptying by scintigraphy may be the result of intervening factors such as a smooth muscle disorder, electro-mechanical dissociation, pylorospasm or incoordination of the antrum and duodenum[20,29]. Hypergastrinemia found in hyperthyroidism may also influence gastric and intestinal motility[30].

Intestine and colon

Appetite increase is common but may not be adequate to maintain weight in severe disease[31]. Up to 25% of patients with hyperthyroidism have mild-to-moderate diarrhea with frequent bowel movements[22,32]. Some degree of fat malabsorption is usually present and may reach 35 g/d[33]. Intestinal hypermotility in thyrotoxicosis reduces small bowel transit time, especially when diarrhea is present[18]. Increased appetite and excessive fat-rich food intake may contribute to excessive fecal fat[34]. Moreover, diarrhea may be related to a hypersecretory state within the intestinal mucosa[22,35]. The adrenergic system may contribute to diarrhea as suggested by correction of transit in hyperthyroid patients treated with the β-adrenergic antagonist propranolol[36]. A reduction in mixing of food with digestive secretions may also contribute to decreased fat absorption. Alterations in intestinal absorptive function are still a matter of debate, as absorption may be increased for glucose[34,37] but decreased for calcium[38]. Anorectal physiology is impaired in hyperthyroidism; when compared to controls, mean anal resting and squeeze pressures are lower as is the rectal threshold of sensation[39].

Liver

Increases in aspartate aminotransferase and alanine aminotransferase in 27% and 37%, respectively, of hyperthyroid patients have been reported[40]. These disturbances are attributed to a hypoxic state with disproportionately increased liver activity compared to blood flow[41]. Mild elevation of alkaline phosphatase is encountered in up to 64% of patients with hyperthyroidism[42-44]. This elevation is not specific to the liver since a high turnover in bones may contribute. Elevations of γ-glutamyl transferase and bilirubin do not exceed 20% of normal values[44]. Increases in liver enzymes and hepatic injury related to anti-thyroid therapy is well documented[45]. Mild histological changes are common[46], but cases of fulminant hepatic failure with centrizonal necrosis have been described[46,47]. Long term untreated hyperthyroidism can ultimately lead to cirrhosis[48]. Quantitative 99mTc-HIDA cholescintigraphy in hyperthyroid rats without a gallbladder showed accelerated bile flow to the duodenum[21].

Hyperthyroidism and associated gastrointestinal diseases

Ch'ng *et al*[6] found that patients with Grave's disease were at a 5-fold added risk of developing celiac disease when compared to sex- and age-matched controls. In such cases, celiac disease may contribute to diarrhea and malabsorption. Thyrotoxicosis has been reported in 3.8% of patients with ulcerative colitis while the incidence of ulcerative colitis in hyperthyroid patients varies around 1%[17]. Thyroid disease may exacerbate ulcerative colitis symptoms or alter the response to therapy. Moreover, a positive correlation between Grave's disease and ulcerative colitis has been reported[12], but a common autoimmune origin could not be proven[11]. Isolated instances of an association between Grave's disease and Crohn's disease have been reported, but a common pathogenesis is still to be identified[16]. Primary biliary cirrhosis in association with hyperthyroidism is extremely rare and has only described as isolated case reports[8]. One study showed a prevalence of pernicious anemia of 5% in thyrotoxic patients, mainly resulting from Grave's disease[49], but parietal cell antibodies have been found in up to 30% of patients[50].

HYPOTHYROIDISM

Hypothyroidism occurs mostly secondary to an autoimmune disease or as a consequence of therapy for hyperthyroidism. It manifests throughout the body with decreased metabolic functions. It is biochemically characterized by the accumulation of glycosaminoglycans, mainly hyaluronic acid, in soft tissues[51]. Interstitial edema predominating in the skin and muscles (including the heart and intestinal muscular layer) will follow. Clinical presentation of the disease is related to the severity of the disease (biochemical derangement) but harbors significant individual variation[52]. Gastrointestinal manifestations are not rare and involve different digestive organs.

Esophagus and stomach

Severe hypothyroidism may lead to disturbances in esophageal peristalsis. When the proximal portion is involved, myxedema causes oropharyngeal dysphagia[53] while esophagitis and hiatal hernia occur when the

distal esophagus is altered[22,54]. Esophageal motility disorders, reduced velocity and amplitude of esophageal peristalsis and a decrease in lower sphincter pressure all contribute to dysphagia[55]. Although it represents an extremely rare cause of dyspepsia, hypothyroidism should be investigated when all exploratory methods are negative[56]. A gastric myoelectrical study led by Gunsar *et al*[19] showed a positive correlation between dyspepsia and hypothyroid scores. Additionally, gastric dysmotility is significantly more frequent in hypothyroid patients and is a result of muscle edema and altered myoelectrical activity[57]. Despite a few contradictory results[58], the hypothyroid state seems to delay gastric emptying[19,59]. Phytobezoar due to hypothyroidism has also been reported[60]. Achlorhydria in hypothyroidism may be related to subnormal serum gastrin[61]. Finally, hypothyroidism is associated with a decrease in duodenal basal electrical rhythm[62].

Intestine and colon

Appetite is usually reduced, but weight gain may reach 10% because of fluid retention[31]. Vague abdominal discomfort and bloating may be erroneously attributed to functional bowel disease[2]. The effect of hypothyroidism on the gastrointestinal tract seems to be multifactorial with possible alterations in hormone receptors, neuromuscular disorders and myopathy caused by infiltration of the intestinal wall. Reduction of peristalsis in hypothyroidism is the main pathophysiologic process[62], and constipation remains the most frequent gastrointestinal complaint[22]. Up to 15% of patients have fewer than 3 bowel movements weekly[2]. Moreover, thyroid hormone deficiency may influence transepithelial flux transport by inhibiting Cl^-/HCO^{3-} anion exchange with a subsequent effect on intestinal motility[35]. Although rare, severe cases of hypothyroidism lead to ileus and colonic pseudo-obstruction with fecal impaction and megacolon[63,64]. Inadvertent surgery in these situations is harmful and may be lethal[5]. Absorption of specific substances may be decreased but the total quantity absorbed is usually normal or increased due to an extended time in bowel transit[31,65]. Diarrhea in the hypothyroid state is mainly the result of increased bacterial growth secondary to bowel hypomotility[66,67]. Exceptionally, hypothyroidism may be the cause of gastrointestinal bleeding refractory to usual treatments[68], most probably by means of acquired coagulopathy[69]. Deen *et al*[39] found that the anorectal physiology is altered in hypothyroid states. While maximal anal resting and squeeze pressures are normal, the threshold for rectal sensation is higher and the maximal tolerable volume is diminished when compared to controls.

Liver

Liver function tests are mildly disturbed in almost 50% of patients with hypothyroidism despite normal histological findings[22]. Decreased hepatic metabolism in hypothyroidism is reflected by reduced oxygen consumption[70] and causes a significant decrease in gluconeogenesis[71] and urea nitrogen production[72]. Myxedema ascites in hypothyroidism is rare and may be a long-standing overlooked and/or isolated sign of the disease[73]. The serum-to-ascites albumin gradient is usually > 1.1 g/dL with a high protein content[4,73]. Although considered to be the result of hypothyroidism-related chronic right-heart failure[74,75], it is mainly attributed to increased permeability of vascular endothelium[4,76]. Patients with a common bile duct stone and gallbladder stone have, respectively, 7-fold and 3-fold increases in the frequency of hypothyroidism[77]. This may be related to the triad: hypercholesterolemia, hypotonia of the gallbladder and reduced bilirubin excretion. Experiments in rats confirmed a thyroxine effect on bile composition[78,79], decreased hepatocytic bile salt excretion in hypothyroid state[80] and relaxation of the sphincter of Oddi[81]. Moreover, Laukkarinen *et al*[21] confirmed that bile flow to the duodenum was reduced in hypothyroid rats.

Hypothyroidism and associated gastrointestinal diseases

Compared to the general population, patients with autoimmune thyroiditis have an almost 5-fold increased risk of developing celiac disease[14,15,82,83]. Valentino *et al*[7] showed that as many as 43% of patients with Hashimoto's thyroiditis carry cellular markers for celiac disease. The prevalence of thyroid antibodies is extremely high in patients with pernicious anemia (57%)[13], and the prevalence of overt pernicious anemia among patients with primary hypothyroidism is 12%[31]. An association between hypothyroidism and primary biliary cirrhosis is well documented and ranges from 5% to 20%[9,10,84]. Among patients with primary biliary cirrhosis, antithyroid antibodies were present in 20%[10]. The coexistence of Hashimoto's thyroiditis and Crohn's disease is rare and the etiological background remains to be elucidated[16,85].

CONCLUSION

Dysthyroidism, whether in excess or deficiency, has clinical manifestations within different portions of the digestive tract and viscera. Whether these are related to hormone level disturbances alone or are associated with a specific thyroid disease, the underlying pathophysiology is often complex and not yet fully elucidated in current studies. Although most frequent manifestations are well known, some situations are often underdiagnosed, leading to serious illness and death.

Digestive diseases related to thyroid hormone abnormalities or associated with particular thyroid diseases must be recognized by most, if not all practitioners. Much research requires to be performed in order to add to our understanding of the scientific background of the older empirical works.

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TOPIC HIGHLIGHT

Dr. Shahid A Khan, *Series Editor*

Gastroenterology in developing countries: Issues and advances

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Abstract

Developing countries shoulder a considerable burden of gastroenterological disease. Infectious diseases in particular cause enormous morbidity and mortality. Diseases which afflict both western and developing countries are often seen in more florid forms in poorer countries. Innovative techniques continuously improve and update gastroenterological practice. However, advances in diagnosis and treatment which are commonplace in the West, have yet to reach many developing countries. Clinical guidelines, based on these advances and collated in resource-rich environments, lose their relevance outside these settings. In this two-part review, we first highlight the global burden of gastroenterological disease in three major areas: diarrhoeal diseases, hepatitis B, and *Helicobacter pylori*. Recent progress in their management is explored, with consideration of future solutions. The second part of the review focuses on the delivery of clinical services in developing countries. Inadequate numbers of healthcare workers hamper efforts to combat gastroenterological disease. Reasons for this shortage are examined, along with possibilities for increased specialist training. Endoscopy services, the mainstay of gastroenterology in the West, are in their infancy in many developing countries. The challenges faced by those setting up a service are illustrated by the example of a Nigerian endoscopy unit. Finally, we highlight the limited scope of many clinical guidelines produced in western countries. Guidelines which take account of resource limitations in the form of "cascades" are advocated in order to make these guidelines truly global. Recognition of the different working conditions facing practitioners worldwide is an important step towards narrowing the gap between gastroenterology in rich and poor countries.

Key words: *Helicobacter pylori*; Developing countries; Gastrointestinal diseases; Health care delivery; Practice guidelines

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INTRODUCTION

Despite political rhetoric, foreign aid, and increased global wealth, the disparity between the developing and developed world is more evident than ever before.

Recently, the economic successes of China and India have lessened poverty for millions of people. Nevertheless, these countries aside, international inequality in income has continued to rise over the past two decades[1].

Nowhere is this inequality clearer than in the arena of health. A child born in Angola in 2006 has a 26% chance of dying before its fifth birthday. In the UK, that risk is 0.6%. In 2006, the life expectancy for a woman from the United States of America was 80 years. If she was instead living in Zambia, her life expectancy declines to just 43 years[2].

In this review, we examine some of the main challenges in developing countries, and discuss potential and existing solutions. Our definition of developing countries is that set out by the International Monetary Fund; "countries with low levels of output, living standards, and technology; per capita GDPs are generally below $5000 and often less than $1500"[3]. The converse, developed countries, will be referred to as "western countries" for clarity. In common practice, these include most of Europe, North America, Japan and Australasia[3] (Figure 1).

The focus of the review will be on gastroenterological problems which head the global disease burden. Although challenges such as war, inadequate water and sanitation, and economic failure all undoubtedly impact on global health, it is beyond the scope of this article to discuss these factors in detail. Moreover, whilst problems such as unstable governments, sectarian violence, and environmental catastrophe undeniably compound health issues, they are by no means confined to developing countries.

In the first part of this review, we will focus on three significant areas of gastroenterological disease which highlight particular problems in developing countries: diarrhoea, hepatitis B and *Helicobacter pylori* (*H pylori*).

The second part of the review will consider the implementation of clinical services in developing nations, encompassing the health workforce, endoscopy services, and the relevance of resource-blind guidelines.

GASTROENTEROLOGICAL DISEASE BURDEN OF DEVELOPING COUNTRIES

Diarrhoeal diseases

The global burden of diarrhoeal diseases outweighs any of the more complex diseases seen in gastroenterology clinics. Every year, there are an estimated 1.5 billion episodes of diarrhoea worldwide[4]. These episodes result in the deaths of approximately 2.2 million people, mostly children in developing countries[4]. This mortality rate has improved from the early 1980s, when diarrhoea is estimated to have caused 4.5 million deaths in children alone[5]. However, it is still the third leading cause of death in under-5 years old, after neonatal causes and pneumonias[6].

Developing countries bear the brunt of this burden. Diarrhoea causes 17.9% of deaths in low-income countries compared to 1.6% in high income countries[6]. Most of these cases are due to the lack of safe water, sanitation and hygiene. Only 34% of people in low-income countries have access to adequate sanitation[6]. As mortality rates from diarrhoea are now so low in western countries, the scale of disease is often expressed in terms of financial costs instead: hospitalisation rates and doctors' consultation time[7]. However, these can be overused resources in the West, and are thus poor comparison measures between countries.

Figure 1 Distribution of countries as per International Monetary Fund (IMF) definitions of economic development (IMF statistical database[3]: reproduced with kind permission of IMF).

Diarrhoeal diseases are caused by a wide variety of pathogens. In 1991, the World Health Organization (WHO) performed a case-control study of the aetiology of diarrhoea in children under 36 mo of age, in five countries: China, India, Mexico, Myanmar and Pakistan. The pathogens most strongly associated with disease were rotavirus, Shigella species and enterotoxigenic *Escherichia coli*[8]. These enteric pathogens, with cholera and typhoid fever, have been identified as the highest priorities for vaccination development by WHO[9].

Diarrhoeal episodes are usually acute and self-limiting. However, they can cause fluid and electrolyte loss from the small intestine so severe that it results in death from dehydration. In some cases, diarrhoea can become persistent: usually defined as lasting at least 14 d[10]. There is evidence that persistent diarrhoea in children can lead to malnutrition[11,12], growth stunting[13,14], and effects on cognitive function[15,16]. A Brazilian study found that children with persistent diarrhoea in the first 2 years of life scored significantly lower on intelligence tests at age 6-10 years, even when controlling for maternal education and helminthic infection[16].

In the late 1980s, oral rehydration therapy (ORT) transformed the management of acute diarrhoea. Physiological studies conducted during the 1950s and 1960s identified the co-transport of sodium and glucose in the small intestine[17-19], which were then harnessed into the oral rehydration solution (ORS) developed at the International Centre for Diarrhoeal Diseases Research in Bangladesh in 1968[20]. WHO adopted and started distribution of a standard ORS in 1975, and set up the WHO Programme for Diarrhoeal Control in 1979[21].

ORT has been heralded as one of the most important therapeutic advances of the past century and has undoubtedly contributed towards the reduction in global child mortality rates described above[22,23] (Figure 2). However it has not reduced the morbidity associated with diarrhoea. Neither stool volume nor

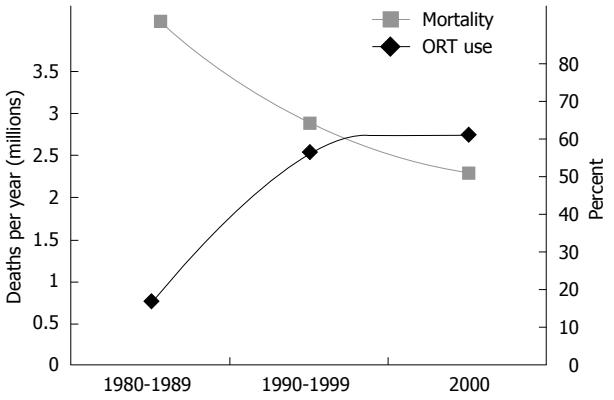

Figure 2 Association between coverage of oral rehydration therapy (ORT) use and mortality rates from diarrhoea in selected countries (from Podewils *et al*[23], 2004. Reproduced with kind permission of Elsevier).

duration of illness are significantly reduced with ORT use, and there may even be a paradoxical increase in stool volume. Therefore, further research has been performed on modifying ORT formulations, including the development of amino acid-containing, starch-based, and reduced sodium solutions. Glutamine, an amino acid, stimulates sodium absorption in experimental models of cholera and rotavirus diarrhoea[24-27]. However, there is conflicting evidence on the efficacy of glutamine containing ORT[20,28], and it is not recommended over WHO ORS at present. The rationale for starch-based solutions (either rice or cereals) is that the polysaccharides would provide more glucose at the intestinal mucosa without the large osmotic load of glucose-based formulations. In addition, they provide nutrition early in the illness. Cereal-based formulations have been shown to reduce total fluid requirements and duration of illness, and are recommended over standard ORS in patients with cholera (but not for non-cholera diarrhoea)[28]. Although cereal-based ORT should be more accessible in rural locations, one study showed that mothers found it more time-consuming to prepare and used standard ORT in preference[29]. In 2003, WHO modified its ORS formulation to contain a reduced amount of sodium and glucose. This hypotonic solution has been associated with less vomiting, decreased stool volume, and reduced need for intravenous fluids[30], and has been recommended for patients with non-cholera diarrhoea. However, concerns have been raised that exclusion of cholera can be difficult in under-resourced areas and use of this formula will lead to hyponatremia in these patients[31]. Clearly, there is still research to be done into the definitive formulation of ORT.

Despite the efficacy of ORT, uptake in developing countries can be variable[32,33]. Difficulties include remembering the correct quantities of ingredients involved in preparing an ORT and high levels of illiteracy[34-36]. Continued effort is required to provide ongoing education at a community level in order to bring about long-term changes[37-40].

More recently, it has been shown that zinc deficiency complicates a significant proportion of diarrhoeal cases[41]. Zinc is not stored in the body, and may be lost from the intestine during diarrhoea[42]. It has a role in immune function[43], however the physiological mechanism linking zinc deficiency with diarrhoea has not yet been elucidated. Several meta-analyses have shown that zinc replacement in acute and persistent diarrhoea reduces both the duration and severity of diarrhoea[43,44], and short (14 d) courses prevent further diarrhoea for 2-3 mo[45]. The WHO has recently recommended that zinc supplementation should be given to all children with acute diarrhoea persisting for at least 14 d[29]. Zinc supplementation has also been shown to significantly reduce the duration of lower respiratory infections[46], the second largest cause of child mortality worldwide.

Universal clean water, hygiene, and sanitation would be the ultimate solution to the global burden of diarrhoea, however in their continued absence, considerable interest has been shown in a more immediate intervention to prevent diarrhoea: vaccination. Of all the pathogens mentioned above, rotavirus is the leading cause of severe diarrhoea in children worldwide. By age 5 years, virtually all children will have been infected by rotavirus, and one in 293 children will have died from it. More than 80% of deaths from rotavirus infection occur in developing countries[47,48]. It also causes a significant financial burden in western countries. Each year in the United States, there are more than 400 000 consultations and up to 70 000 hospital admissions due to rotavirus[49]. Therefore, there has been substantial investment in the development of vaccines against rotavirus infections, both for western and developing countries.

In 2000 and 2001, China introduced a monovalent lamb-derived live attenuated oral vaccine[50]. However, the efficacy of this vaccine is not known, as it was not tested against placebo in the final stages. The focus of research in other countries has been on developing a vaccine against multiple rotavirus serotypes, in order to provide heterotypic protection[48]. The first multivalent live oral reassortant vaccine developed was RotaShield, which was highly effective in field trials in the United States, Finland and Venezuela[51-54]. It was included in the USA immunisation programme in 1998[55], however several cases of intussusception were reported, and the vaccine was subsequently withdrawn[56]. This risk was estimated to be only one per 10 000 vaccinated infants[57], however trials in Ghana, Bangladesh, and India were also stopped at that time, and it was thus not possible to do a risk-benefit analysis for developing countries[48]. Two further rotavirus vaccines have since come onto the market; Rotateq, a human-bovine live-attenuated oral vaccine, and Rotarix, a human live-attenuated oral vaccine. Trials in medium and high-income countries have produced good efficacy results for both these vaccines[58,59], however more trials are needed in developing countries. A large commitment to funding from donor countries will also be required to further reduce global child mortality from diarrhoea.

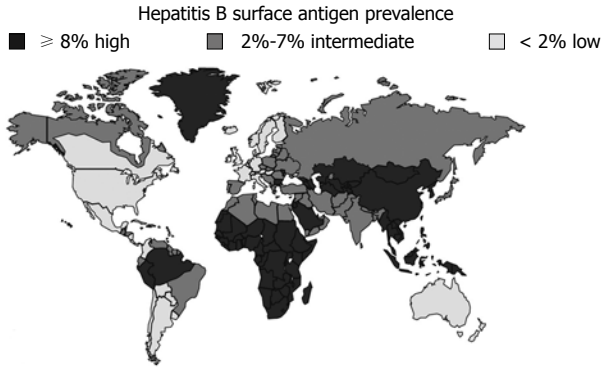

Figure 3 Geographical distribution of the prevalence of chronic hepatitis B virus infection, 2002 (from Mast *et al*[67], 2004. Reproduced with kind permission of Elsevier).

Hepatitis B virus (HBV)

Hepatitis B is the foremost hepatological health problem in the developing world. Up to two billion people worldwide have serological evidence of past or present HBV infection, and 360 million have chronic infection[60,61]. Through its long-term sequelae of liver cirrhosis and hepatocellular carcinoma (HCC), HBV causes 500 000-700 000 deaths each year[60] and is an accessible target for cancer prevention on a massive scale. Although hepatitis C virus (HCV) is increasing in importance, particularly in the western world, HBV is still estimated to account for 50%-55% of HCC worldwide compared to 25%-30% for HCV[62-64].

HBV varies in its prevalence worldwide. Countries can be divided by their level of endemicity, which is based on the percentage of the general population that is seropositive for HBsAg (chronic carriers). Countries with high endemicity have more than 7% seropositivity levels, intermediate 2%-7%, low 0.5%-2% and very low endemicity countries have < 0.5% seropositivity[65,66] (Figure 3[67]). Developing nations make up the bulk of high endemicity countries, including much of sub-Saharan Africa and South East Asia. Notable pre-vaccination examples include the Gambia, where 36% of children were chronic carriers[68], and Taiwan, with 15%-20% chronic carriage in the general population[69-71].

Of particular relevance to developing countries, the likelihood of acquiring chronic HBV infection depends on the age of acquisition of the virus[60,72,73]. For children under 1 year, the risk of chronic infection is 90%. For 1-5 years old, the risk is 30% and for children older than 5 years and adults, the risk decreases to only 6%. This feature accounts for most of the disparity in prevalence outlined above.

The main modes of transmission also vary between countries of high and low endemicity. In high endemicity countries, and in stark contrast to the West, perinatal and horizontal transmission (exposure from close household contacts or play with other children) are the dominant modes[66,74]. In these countries, 70%-90% of the population show serological evidence of previous or current HBV infection. In lower endemicity countries, HBV transmission is mainly limited to high risk groups, such as intravenous drug users and healthcare workers, or is acquired sexually. Although not the main transmission mode, healthcare-acquired infections can assume greater importance in developing countries due to lack of resources for disposable equipment and sterilisation, or lack of awareness of infection control practices[65,75]. However, blood products in most parts of the world are now screened for HBsAg[76].

Chronic infection is responsible for the main burden of disease associated with HBV. Approximately 20% of chronic carriers will die prematurely from cirrhosis leading to liver failure or HCC[77]. Although therapies are available which can suppress HBV replication or modulate the immune reaction, these are expensive and not widely available in much of the developing world. There is currently no therapy which results in virus eradication.

Therefore, the emergence of a plasma-derived vaccine against HBV in the early 1980s was a significant event. This was the world's first cancer prevention vaccine and the first vaccine to prevent a sexually transmitted disease, both functions now echoed by the recently licensed human papillomavirus vaccines. Most current vaccines are produced by recombinant technology[65], and the vaccine prevents HBV infection in 90%-100% of people who produce sufficient antibody responses[78]. It is also highly effective as post-exposure prophylaxis in cases of possible perinatal transmission, even where HBV immunoglobulin co-administration is not possible[79]. Current consensus is that booster doses are not necessary to maintain immunity[60]. Finally, although susceptible to freezing, present vaccines are heat stable, a great advantage in developing countries where access to cold storage facilities is often difficult[60].

In 1992, WHO's Global Advisory Group of the Expanded Programme on Immunization recommended that all highly endemic countries included hepatitis B vaccination into their national childhood immunisation programs by 1995, and all other countries by 1997[80,81]. As of 2006, more than 160 countries had implemented universal hepatitis B vaccination[82]. Several western countries with very low endemicity, such as the United Kingdom, have chosen to pursue a policy of targeted vaccination of high-risk groups rather than universal vaccination[83].

In countries which implemented universal childhood vaccination early on, such as Taiwan, the Gambia, and Malaysia, HBV vaccination was found to be very effective, both in terms of disease prevention and health costs[66,84]. The ultimate goal of these programmes is to prevent the long-term consequences of cirrhosis and HCC, therefore it will be some years before a complete evaluation can be carried out on the first vaccinated cohorts. However, indicators such as HBV seroprevalence and hospital records of acute HBV infections, provide early evidence of their successful impact.

In Malaysia, where universal vaccination was introduced in 1990, HBsAg seroprevalence among children aged 7-12 years decreased from 1.6% in 1997 to

0.3% in 2003[85]. In the Gambia, where HCC is the most common tumour in men[86], vaccination was introduced progressively between 1986 and 1990. Childhood HBsAg seroprevalence has since decreased from 10% in 1986 to 0.6% in 1991[87-89]. Similar declines have been shown in Senegal, China, Indonesia, and Thailand[90].

The best example of the effectiveness of a HBV vaccination programme is probably Taiwan, which had very high levels of chronic carriage in the pre-vaccination era[91]. Over 90% of the population under the age of 40 years had been infected by HBV[92]. Universal infant vaccination was introduced in Taiwan in 1984, one of the first regions to do so[91,93]. 15 years after implementation, HBsAg seroprevalence amongst children 1-15 years decreased from 9.8% in 1984 to 0.7%[94]. In addition, the incidence of fulminant hepatitis amongst infants also decreased. The average mortality from fulminant hepatitis in infants between 1975 and 1984 (pre-vaccination) and from 1985-1998 (post-vaccination) was 5.36 and 1.71 per 100 000 infants, respectively[95].

These evaluations show that HBV can be effectively prevented through a universal vaccination programme. As humans are the only known natural host of the virus, it is feasible that vaccination could eradicate HBV from the world. The major obstacle to global coverage of HBV vaccination is funding. Although the cost of monovalent HBV vaccines had decreased from approximately US $3.00 per dose in 1990 to US $0.30 per dose in 2001[96]. the cost is still higher than the other vaccines included in the extended programme on immunization (e.g. measles, oral polio) which cost between US $0.06 to 0.10 per dose. Several manufacturers have produced combination vaccines containing hepatitis B antigen which allow the addition of hepatitis B vaccine into existing childhood immunisation programmes, however again these are expensive and beyond the capacity of many developing countries.

The Global Alliance for Vaccines and Immunization (GAVI) was founded in 1999 to address this funding gap. GAVI is a consortium between WHO, the World Bank, UNICEF, the Bill and Melinda Gates Foundation, governments of both developing and developed nations, and the vaccine industry[97]. By 2007, it had provided funding for 67 countries out of 69 eligible for support towards the introduction of HBV vaccination programmes[98]. As a result, global three dose vaccine coverage has nearly doubled since 1999 (Figure 4[82]). However, the millions currently infected with HBV in the developing world carry an impending disease burden that will be substantial in the near future.

H pylori infection

It is estimated that 50% of the world's population is infected by *H pylori*[99]. Although most infections are not associated with clinical disease, a significant proportion will go on to develop some of the commonest problems in gastroenterology: gastritis, peptic ulcer disease, and gastric cancer[100-103]. Although less than one percent of infected persons will develop gastric cancer, this is the fourth most common malignancy in the world[104]. It is

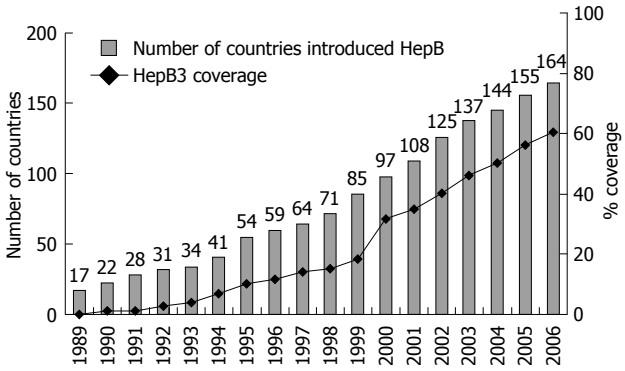

Figure 4 Graph showing number of countries who have introduced hepatitis B vaccination programme and global infant three dose vaccine coverage ("HepB3") (reproduced with kind permission of World Health Organisation).

Table 1 Estimated *H pylori* infection prevalence globally (reproduced with kind permission of World Gastroenterology Organisation)

Country/region	Estimated prevalence of *H pylori* infection (%)
Mexico, Central/South America	70-90
Africa	70-90
Asia	50-80
Eastern Europe	70
Western Europe	50-30
United States and Canada	30
Australia	20

these strong disease associations which establish *H pylori* infection as a leading gastroenterological public health problem.

There are distinct differences in the pattern of *H pylori* infection between developing and western countries. The prevalence of infection in the West has been declining for some years, however it is still very high in developing countries[99], with the majority of the global burden of infection found here (Table 1[105]). This is not surprising, given that risk factors for the infection include low socio-economic status, crowded living conditions, several children sleeping in one bed, large number of siblings and unclean water-all conditions common in the developing world[106-110]. Low education levels have also been positively associated with *H pylori* infection in several studies[111-113]. Prevalence levels in developing countries are therefore associated with the circumstances induced by poverty-and are unlikely to follow the decreasing trend of the western world without alleviation of this factor.

Another marked difference between *H pylori* infection in developing and western countries is the age at acquisition of the infection. Individuals tend to be infected much younger in developing countries than in western countries. In many developing countries, the prevalence of infection exceeds 50% by 5 years of age[114]. In a study of Bangladeshi children, the prevalence of *H pylori* infection was 42% by 2 years of age. Another study of Gambian children using the

diagnostic ^{13}C-urea breath test (UBT) found prevalence levels of over 75% in the second year of life[115] (although very young ages may produce false positive results in the UBT[116]). Comparative studies in western countries show prevalences ranging from 6% in Finland[117], 11% in Scotland[118], 13% in Germany[119], and 23% in Italy[120,121].

Epidemiological studies support person-to-person transmission, which is likely to be *via* faecal-oral and oral-oral routes[122]. The oral-oral route is supported by the finding that pre-mastication of food (the chewing of food by mothers before feeding their babies) is associated with increased prevalence of *H pylori* in infants[106,123,124]. Pre-mastication of food is a common practice in both South-East Asia and Africa. Water sources have also been implicated as a potential mode of transmission, possibly through faecal contamination. An early Peruvian study showed that children living in households with a municipal water supply had a markedly higher risk of *H pylori* infection compared to those who had access to well water[125]. This was supported by findings from a study in Bolivia which found that children living in families using containers which prevented direct contact with this drinking water were significantly less likely to have *H pylori* infection compared to families without this container[126]. Iatrogenic transmission through contaminated endoscopes has been documented both in western and developing countries[127-129], and may be a particular problem in those countries where lack of resources hinders full disinfection procedures[130].

Worldwide, 90% of duodenal ulcers and up to 70% of gastric ulcers are associated with *H pylori* infection[100]. However, peptic ulcer disease is more likely to be reported in western countries, whereas gastric carcinoma is the more common disease association in developing countries[131] In 1994, the International Agency for Research into Cancer designated *H pylori* as a Class I Carcinogen[132].

It is hypothesized that the global variance in disease presentation is related to the age of acquisition of *H pylori* infection[101,122,133]. Infections acquired early in childhood, as in most developing countries, may cause persistent chronic low-grade inflammation which is linked with gastric cancer. Conversely, infections acquired later in life or in adulthood are associated with a more acute inflammatory response and thus ulcer disease.

However, differing incidence rates of gastric cancer globally has led to the description of the "African enigma": that despite a high prevalence of *H pylori* infection, this region has a relatively low incidence of gastric cancer[114,134]. However, this description has been disputed as the average life expectancy on the continent is low (51 years in 2006)[6]. Therefore, individuals may die of other causes before an age at which gastric cancer would become apparent. Indeed, a recent review by Agha *et al*[135] of endoscopy studies carried out on the continent found that *H pylori*-associated peptic ulcers and gastric cancer occurred at similar rates to Western levels.

H pylori infection can have significant sequelae in children in developing countries in addition to the long-term effects of chronic inflammation. Acute *H pylori* infection induces hypochlorhydria, which can be persistent[136-138]. Hypochlorhydria is associated with an increased risk of diarrhoeal diseases, as the gastric acid barrier is effective against many enteric pathogens[139,140]. Therefore, children infected with *H pylori* may be more likely to suffer from diarrhoeal diseases, both acute and persistent[140-143]. In fact, similar findings of malnutrition and decreased growth to those described above for diarrhoeal diseases have been shown in children infected with *H pylori*[142,144-146].

Spontaneous remission of *H pylori* is rare. For symptomatic infections, eradication is usually achieved by a course of antibiotics (typically clarithromycin, amoxicillin or metronidazole) combined with a proton-pump inhibitor[147]. There are other regional guidelines which recommend specific combinations, some of which are directed towards cost issues[148,149]. However, low-cost options may not be as effective as more expensive regimes and may necessitate repeat treatment, leading to higher costs overall[105]. In addition, although eradication may be achievable in western countries with a 7 d regime, treatments of 14 d may be required in developing countries. A study from Brazil showed eradication rates of only 50% if therapy was less than 10 d[150]. Subsequently, a meta-analysis showed a 12% higher eradication rate for 14 d *versus* 7 d regimes[151]. This must be balanced against the likelihood of patient compliance to a complex regime of drugs for 2 wk.

Unfortunately, eradication has been increasingly affected by antibiotic resistance levels worldwide. Many antibiotics are available "over the counter" in developing countries, i.e. not subject to prescription from a doctor. Metronidazole is also used to treat common enteric infections such as amoebiasis and giardiasis: often empirically. Metronidazole resistance is an increasing problem worldwide, although may not affect eradication as much as clarithromycin resistance[114,147]. Antibiotic resistance is the main reason for treatment failure. If sensitivity testing is available, this should guide choices for local first-choice and rescue therapy[105,147].

Re-infection rates after eradication can be as low as 1% in western countries[114]. Given the much greater prevalence of *H pylori* infection in developing countries, it is not surprising that re-infection rates there are also markedly higher. Studies from Chile and Bangladesh have found re-infection rates of around 13%[152,153]. It was difficult, however, to distinguish re-infection from recrudescence in these studies[114].

These issues highlight the need for a vaccine against *H pylori*. As for HBV, there is a need for both preventative and therapeutic vaccines, with the preventative vaccine used primarily on young children in high prevalence areas. Rupnow *et al*[154] modelled the population impact of a prophylactic vaccine. For a typical developing country, they found that with continuous vaccination, *H pylori*-attributable gastric cancer would decrease from 31.8 per 100 000 to 5.8 per 100 000 by 2100. Unfortunately, the current status of *H pylori* vaccines is disappointing. A number of trials have been conducted examining the safety and immunogenicity of various

formulations including recombinant urease, killed whole cells, and live vectors expressing *H pylori* antigens[155]. However, the vast majority of these showed low immunogenicity. Further research is needed to elucidate the mechanism of immune protection and the role of adjuvants.

In conclusion, *H pylori* infection is recognised as a significant public health problem in developing countries. Both antibiotic resistance and re-infection rates threaten the efficacy of existing eradication therapy. Definitive treatment in the form of both prophylactic and therapeutic vaccines is urgently needed in order to alleviate the burden of disease associated with this bacterium.

DELIVERY OF CLINICAL SERVICES IN DEVELOPING COUNTRIES

The first part of this review described three major gastroenterological diseases in the developing world: diarrhoeal disease, hepatitis B, and *H pylori*. Given this burden of gastroenterological disease, specialists in gastroenterology and hepatology are particularly important in developing countries. However, as in most specialities, there is a shortage of trainees.

Mass education and vaccination programmes against diarrhoeal infections, HBV, and potentially *H pylori*, all require immense resources for delivery. Dissemination of the ORT message requires health workers trained in education and prepared to work in remote areas. The consequences of HBV and *H pylori* infection are optimally treated by specialist referral and endoscopy services. However, one of the most limiting factors in the delivery of clinical services in developing countries is the severe lack of trained healthcare personnel.

Health workers

WHO estimates that there is a global shortage of 4.3 million healthcare workers. Africa alone needs an estimated 1.5 million more health workers in order to provide just basic health services[156]. For many years, the strengthening of national health systems and training of personnel have not been included as part of international aid programmes[157]. Staff have been mainly trained intensively for a particular programme's focus, with little integration into a comprehensive national system of health workers.

The result is an uneven distribution of health workers, inverse to the world's health needs. The Americas, including Canada and the United States, have 10% of the global burden of disease, yet almost 37% of the world's health workers. Sub-Saharan Africa, conversely, has more than 24% of the disease burden, yet has only 3% of health workers and less than 1% of the world's financial resources[156]. Ethiopia, for example, has two doctors per 100 000 population. The UK has more than 230 per 100 000[2] (Figure 5[156]). Fifty-seven countries are estimated to have critical shortages of health workers: 36 in sub-Saharan Africa[156].

Figure 5 Geographical distribution of countries with a critical shortage of health service providers (doctors, nurses and midwives) (reproduced with kind permission of World Health Organisation).

There is also a great imbalance within countries. Many hospitals and medical centres are centred in urban areas, whilst populations of many developing countries are still predominantly rural. In both developing and western countries, highly skilled workers can resist rural postings, as there is a higher quality of life and opportunity for money in the urban areas[157,158]. In Malawi, 85% of the population live in rural areas[2]. However, out of Malawi's 156 public sector doctors, 81 are working in central hospitals[159,160]. This leaves many districts without any doctors at all.

There is a general perception that economic migration by skilled health professionals is the main cause of the global shortage. There has been debate in western countries over the ethics of wealthy, more developed countries with a relative density of health workers accepting highly skilled medical migrants from countries with a severe need and valuable investment in their initial training[161]. It is estimated that the UK has saved some £65 million in training costs between 1998 and 2002 by recruiting Ghanaian doctors[162], whilst Ghana has lost £35 million of its training investment to the UK. However, although migration certainly plays a part, it is not the major factor. If Ethiopia had all the doctors it had trained over the last 30 years, there would still only be approximately ten doctors per 100 000 population[157]. The total number of African-born doctors and nurses working in Organisation for Economic Co-operative and Development countries account for less than 12% of the estimated shortages in Africa[163]. (While migration may not account for overall shortages in medical manpower its effects appear to be especially felt among highly trained specialists. In some developing countries almost 100% of specialists leave the country; most never to return).

There are not enough healthcare professionals being trained in developing countries to sustain the needs of the population. Ethiopia produces about 200 doctors per year for 75 million population[164]. The UK produces 6000 per year for 60 million people. Two thirds of Sub-Saharan African countries have only one medical school, whilst some still have none[165]. This inadequate production of health professionals, combined with the accumulative effects of migration and losses due

to the HIV/AIDS epidemic (44 Malawian nurses died in 1999-44% of the annual number trained)[166] have resulted in the severe shortages seen today.

There are several reasons for this low production rate of health workers. Foremost of these, inevitably, is funding. WHO estimates that it would cost $26.4 billion dollars to train the extra 1.5 million health workers needed for the African region, not including provision for future salary costs[156]. Any extra training or production of healthcare workers would require great motivation by donor agencies and governments to provide sustainable funding. Firstly, there is a high rate of student and teacher attrition. Students often find it difficult to find sufficient funding for tuition fees, even with government subsidies. Students who have received poorer quality secondary education may struggle with a medical course, and there is often little support available. Health worker shortages inevitably affect teacher numbers, with remaining lecturers shouldering an increased workload[157].

Once qualified, new graduates are faced with difficult working conditions. Lack of resources can lead to poor job satisfaction and high levels of HIV seropositivity make practical procedures hazardous[156,158]. There has also been a tendency to focus on pre-service training to produce health workers, compared to postgraduate education. Opportunities for specialisation and career progression are few[167]. For example, in Malawi, only 5% of medical specialists and 8% of paediatric posts are filled[159,160]. Salaries which are not adequate to cover living costs make posts in western countries very appealing. In 2004, a junior doctor in Malawi earned approximately £1900 per annum[160]. Basic annual pay for a pre-registration house officer in the UK is £21 000[168]. Indeed, there may be strong family pressures to take a job overseas and provide valuable remittances for those who remain in the country. Some countries have actively embraced health worker migration as a source of revenue. The Philippines operates a managed migration policy and is now the largest provider of registered nurses working overseas[158]. However, 30 000 nurse posts are currently unfilled in the Philippines[158].

The consequences of all these factors on a medical specialty are amply illustrated by the situation in gastroenterology. The world-wide burden of digestive illness is tremendous; for example, digestive cancers, collectively, are the most common malignant diseases. Furthermore, while infectious diseases, as illustrated in the first section, represent a major and persisting challenge for developing nations, urbanization and westernization now threaten to inflict the gastroenterological problems of the West, such as those related to obesity[169] on these already underserved populations. In addition, in certain developing countries prevalence rates for gastroenterological disorders which are very rare in the West, such as gall bladder and biliary cancer, are high and a public health issue. Yet many African countries possess not a single gastroenterologist. Though some would argue that more basic medical care should be the priority in these countries, the World Gastroenterology Organisation (WGO) would counter that to deprive these countries of the expertise that specialists and sub-specialists can provide is condescending, to say the least. If nothing else, such expertise is needed to assist in health care provision planning. It should come as no surprise, therefore, that the many advances in the field of gastroenterology which have so dramatically advanced patient care and improved mortality and morbidity in the West, have not been evenly bestowed on the world's population; some areas of our planet have barely felt the impact of advances in diagnostics and therapeutics. A very striking example is provided by the failure of the laparoscopic era which has so revolutionized digestive surgery elsewhere to even dawn in many African countries. Lack of resources is certainly a factor but lack of skilled personnel is also contributory. Similarly, in many African countries none save for the most privileged have access to diagnostic services, such as endoscopy and ultrasound, that would be deemed routine elsewhere.

More global attention has been paid to the issue of health worker shortages over the last few years. The World Health Report in 2006[156] was dedicated to the health worker crisis. The Global Workforce Alliance, which is hosted by WHO, was set up later in 2006 in order to collate and implement effective strategies to tackle the shortages. In 2008, the first Global Forum on Human Resources for Health was held in Uganda.

Given the urgency of the problem, there is a consensus that innovative solutions are needed rather than simply increasing the number of medical training places. One approach, which has been successful in several developing countries, has been to shift away from a western-style distribution of health workers. Low- and medium-level workers, such as community workers and nursing auxiliaries, can be more appropriate for the needs of the population rather than dependence on high-level workers such as doctors and nurses[157]. Not only is there a greatly reduced training time for these lower-level workers, but more workers can be produced for the same training investment and salary costs are lower. In addition, these workers do not have internationally recognised qualifications, and therefore are less likely to emigrate[157,158]. Although high-level workers are still needed for supervision, some countries have had great success in achieving basic health coverage with community workers.

In 1994, Pakistan created the Lady Health Worker (LHW) cadre, aiming to train 100 000 female community health workers by 2005[157]. These workers are recommended by their community, usually in rural and urban slum areas, and are trained for 15 mo in the prevention and treatment of common illnesses. An evaluation of the scheme in 2002 found that populations served by a LHW are more likely to adopt antenatal care, receive medical assistance at birth, and use family planning services[170]. In Malawi, there is a high rate of trauma associated with farming and road traffic accidents, but only nine orthopaedic surgeons[171]. Orthopaedic clinical officers (OCO) are specifically

trained over 18 mo to be able to fulfil most of the orthopaedic roles required in rural district hospitals, including the conservative management of fractures and dislocations, and some external fixation methods. Since the programme began in 1985, it has trained 117 OCOs, who now manage an estimated 80% to 90% of the orthopaedic workload in Malawi[171]. Indeed, a reliance on western-style models of health workforces has meant that in sub-Saharan Africa low and mid-level workers make up only 7% of the workforce, compared to around 20% in Brazil and Iran[172].

It has also been commented that western models of medical curricula may not be appropriate for countries with an urgent need for health workers. A 5-year course with a strong focus on basic sciences may be a luxury in developing countries with high levels of communicable diseases and limited resources. The St Paul's Millennium Medical School in Ethiopia was set up by the government as part of its aim to increase the national production of doctors to 1000 per year[157]. Here, the curriculum has been cut down from 5.5 to 3.5 years, with an emphasis on practical skills, in order to better prepare graduates for their 5 years service in rural hospitals (Professor Gordon Williams, Dean of St Paul's, personal communication).

Internationally, there have been several bilateral agreements which aim to promote ethical recruitment in response to criticism of western countries' active recruitment of foreign health workers. The UK-South Africa Memorandum of Understanding was signed in 2003 and aims to decrease the efflux of South African health workers through efforts to offer time-limited placements as alternatives and to promote UK self-sufficiency[173]. Norway is also developing a policy which will invest in health worker development projects, whilst increasing the number of national training places to encourage self-sufficiency[173]. Changes under the new UK career progression scheme Modernising Medical Careers is also likely to have an effect on international recruitment. For all training posts, UK graduates and those from the European Economic Area are prioritised over international medical graduates[174]. Although prompted by efforts to improve NHS stability and secure training places for all UK graduates rather than ethical concerns, this policy is likely to decrease the attractiveness of the UK medical job market abroad.

If health workers increasingly remain in their home country, further training must be made available in order to provide adequate numbers of specialists and promote more advanced skills. For gastroenterology specialist training, the main advocate is the WGO. The current objectives of WGO are enshrined in its mission statement: "to promote, to the general public and health care professionals alike, an awareness of the world wide prevalence and optimal care of digestive disorders through the provision of high quality, accessible and independent education and training", which signals the commitment of WGO to address two challenges: firstly, providing the gastroenterologist of the future with an optimal training and, secondly, and most pertinent to this review, bringing the benefits of digestive health care to those who currently struggle or, indeed, fail to achieve access to it. The primary emphasis of WGO, therefore, is on education and training; these objectives are achieved through three distinctive, though closely inter-related, programmes: Training Centres, Train-the-Trainers, and Global Guidelines (described later in review).

Figure 6 Map showing location of World Gastroenterology Training Centres worldwide (reproduced with kind permission of World Gastroenterology Organisation).

Training Centres most directly address the issue of training specialists in gastroenterology or individuals with additional expertise in gastroenterology to serve previously underserved areas. Each centre represents a direct collaboration between local experts, international faculty and several national and regional societies from Europe and North America to deliver regionally relevant training to those who have limited, or in some cases, no access to such opportunities. Our centres in South Africa, Morocco, Egypt, Bolivia, Pakistan, Thailand, Mexico City and Fiji (Figure 6) provide training of variable duration to several hundred young and aspiring gastroenterologists and digestive surgeons from underserved nations in their region. In some of these instances, such as in the centres in Soweto and Suva, the focus is on providing training opportunities to young doctors from areas where little or no gastroenterological expertise exists (in these cases Sub-Saharan Africa and Oceania, respectively). More recently, WGO has established partnerships with centres in Italy, Chile, Argentina, and Brazil to provide more advanced training opportunities to the young doctor who has already completed basic training.

Activities at these training centres are supported by other linked WGO programmes. Train-the-Trainers courses are uniquely devoted to bringing the very latest in educational techniques to those who will train the gastroenterologists of the future, including those who teach and train at our Training Centres. These networks should be accessible to all who seek to train in our specialty, thereby, ensuring the highest standards of care for those who suffer from digestive disorders through the world.

Clinical services

The limiting effect of the shortage of trained health workers and specialists can be seen in the central clinical service of gastroenterology: endoscopy.

In western countries, endoscopy services have

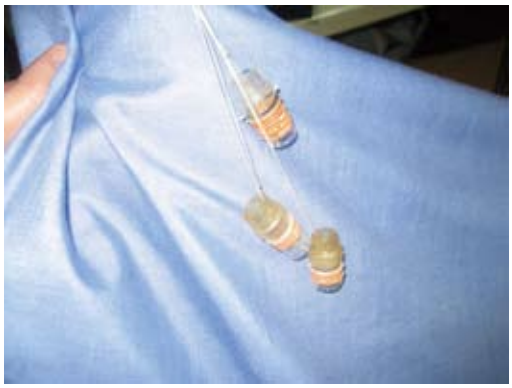

Figure 7 Cut Foley urethral catheter reloaded unto the Opti-vu cap for variceal band ligation at the Endoscopy Unit of Jos University Teaching Hospital, Nigeria.

become such a routine procedure that facilities are readily available, even in some primary care centers. However, in developing countries, services are only available in so-called "centers of excellence" and are rudimentary in most circumstances. They often comprise of direct viewing fibre-optic endoscopes only and are mostly restricted to diagnostic gastroscopies.

This difference is not surprising due to the numerous challenges posed by establishing endoscopy units, including training needs, adequate disinfection facilities and equipment. There is also a lack of awareness amongst most healthcare professionals of the usefulness of therapeutic endoscopy.

While the overall picture looks bleak, there have been some attempts by developing country gastroenterologists to establish endoscopy services. Variceal haemorrhage from portal hypertension is associated with high mortality in most West African countries, due to the lack of endoscopic banding facilities[175]. The Endoscopy Unit at Jos University Teaching Hospital in Nigeria has recently started to perform oesophageal variceal banding and injection sclerotherapy. It costs approximately $300 USD for a single use variceal band ligator, which is vastly prohibitive for most developing country workers. Therefore, the gastroenterologists working at the Endoscopy Unit have modified the normal variceal banding technique by cutting size 14 Foley urethral catheters to size and reloading these on previously used Opti-vu caps (Figure 7). Although not optimal practice, this has reduced the cost to only $30 per session. The modified technique has allowed much greater uptake of the procedure, with improved clinical outcomes. If such interventions become widespread nationally, it has the potential to markedly improve the prognosis in complications of end-stage liver disease, which currently carries a significantly burden in Nigeria.

The training programme for Nigerian gastroenterologists in endoscopic therapies has been bolstered by Royal College of Physicians educational bursaries, which have allowed visits to the UK and reciprocal visits to Nigeria. The Tropical Health and Education Trust also provides training for frontline health workers in the poorest settings, and develops the institutional capacity of local health institutions. This is achieved by focusing on the goals of local health care specialists in individual hospitals, clinics and primary health care projects and offering specialist support and training from UK-based health professionals on a one-to-one basis. Finally, the World Organization of Digestive Endoscopy has set up training centres in Cairo, Egypt and Soweto, South Africa with the aim of improving the management of gastrointestinal disorders in sub Saharan Africa.

It is evident that effective clinical services can exist in developing countries despite being tailored to available resources. Difficulty in adhering to western-defined standards should not necessarily inhibit medical action with benefit to the population.

Guidelines and cascades

Numerous guidelines are produced annually by prestigious medical bodies. The vast majority of these outline "gold standard" practice and are aimed at physicians in resource-rich environments. As such, they are inaccessible and irrelevant for many clinicians in developing countries. As in the case of endoscopy above, many clinicians in developing countries have to "make do" with available resources: in full knowledge that this falls below the gold standard. However, by failing to acknowledge this situation and providing the "next-best option", western guidelines may be preventing the dissemination of knowledge and evidence to its full global audience[176-179].

In order to make guidelines more applicable to differing resource environments, the concept of "cascades" have been developed[177,180]. A cascade is a collection of related diagnostic and treatment options arranged hierarchically in terms of conditions and available resources. Whilst guidelines should continue to summarise best known practice, they could also include alternatives for clinicians with limited funding. These alternatives are usually on the basis of cost, but could also take account of local availability, technology, and infrastructure. Cascades can range from a simple list of options (Table 2) to more complex parallel diagnostic and treatment pathways (Figure 8). In this way, they transform guidelines from being "resource-blind" to "resource-sensitive". Inevitably, cascades are more heavily based on empirical evidence than gold standard options. Research funding is usually spent on trying to improve on best practice-rather than the practicalities of delivery in developing countries. However, with strong involvement from experienced clinicians in developing countries, a consensus is usually reached. More widespread use of cascades in guidelines may also motivate research into the best options for resource-limited services.

Several organizations are now using cascades regularly in their work. The WGO is aiming to include cascades in all of its new guidelines, and has so far published on *H pylori*, acute diarrhoea, treatment of oesophageal varices, colorectal cancer, and hepatitis B[105,181-184]. To aid dissemination, most WGO guidelines are available in at

Table 2 Cascade for treatment of oesophageal varices (reproduced with kind permission of WGO)

Endoscopic band ligation plus octreotide or terlipressin (gold standard)
Endoscopic band ligation alone
Endoscopic sclerotherapy
Balloon tamponade

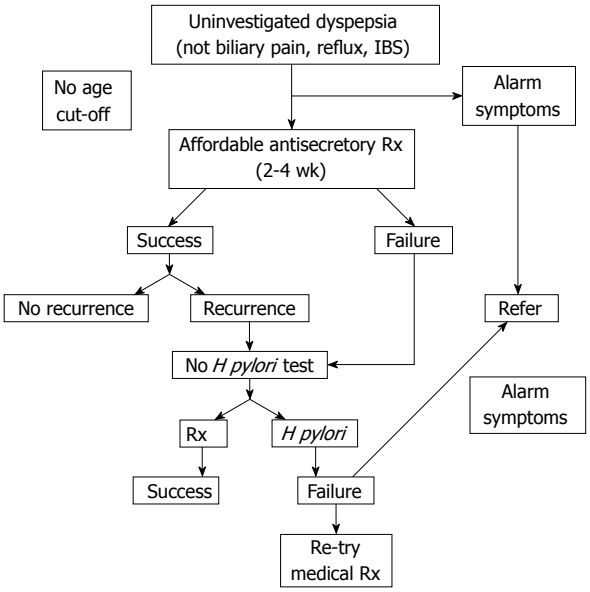

Figure 8 A more complicated cascade for management of dyspepsia in a region with a high prevalence of *H pylori* infection but limited access to endoscopy (reproduced with kind permission of WGO). IBS: Irritable bowel syndrome; Rx: Retreatment; Success: Symptoms resolve; Failure: Symptoms persist.

least four languages; downloads of non-English versions now account for more than 50% of website traffic. The Asian Pacific Consensus on the msanagement of *H pylori* was an early piece of work produced in response to the need for regional guidelines which took account of resource limitations[149]. A programme which uses cascades in response to the urgent need for improved early detection and basic treatment of breast cancer in developing countries is the Breast Health Global Initiative[185,186]. This is an international alliance of health organizations, government agencies, and leading clinicians, which recognises the inflexibility of western-developed screening programmes and aims to produce evidence-based and economically feasible guidelines for medium and low resource regions.

CONCLUSION

The first part of this review has described the disparity between developing and developed countries for three prominent public health problems: diarrhoeal diseases, hepatitis B, and *H pylori*. In this second section, we have considered some of the major obstacles developing countries face in the delivery of gastroenterological services. Increased investment into health workers worldwide is long overdue. Using endoscopy as an example, some of the current difficulties in clinical services implementation in developing countries were highlighted. Finally, guidelines which acknowledge and adapt to the reality of resource limitations would greatly improve information delivery worldwide.

Ultimately, however, perhaps the most important factor needed to improve healthcare in developing countries is the alleviation of poverty. A report in 2001 from the Commission on Macroeconomics and Health[187] showed a negative correlation between the infant mortality rate of a country and the rate of growth of their gross domestic product-the lower the mortality rate, the faster the growth of the economy. An improved economy would allow more investment in sanitation, housing, water quality: all factors which would effectively reduce the prevalence of the diseases discussed above. Until then, however, we must increase recognition of the varying situations facing gastroenterological colleagues worldwide.

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REVIEW

CD74 in antigen presentation, inflammation, and cancers of the gastrointestinal tract

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Abstract

CD74 is a protein whose initial role in antigen presentation was recognized two decades ago. Recent studies have revealed that it has additional functions as a receptor for macrophage migration inhibitory factor and as a receptor for an important human pathogen, *Helicobacter pylori* (*H pylori*). The role of CD74 as a receptor is important because after binding of migration inhibitory factor or *H pylori*, NF-κB and Erk1/2 activation occurs, along with the induction of proinflammatory cytokine secretion. This review provides an up-to-date account of the functions of CD74 and how it might be involved in inflammation and cancer within the gastrointestinal tract.

Key words: CD74; Invariant chain; Cancer; Inflammation; *Helicobacter pylori*; Gastrointestinal tract

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INTRODUCTION

CD74, also known as the invariant chain or Ii, is a non-polymorphic glycoprotein that has diverse immunological functions. The most well-known function of CD74 is regulating the trafficking of class Ⅱ major histocompatibility complex (MHC) proteins in antigen presenting cells. More recently, CD74 expression has been examined in cell types other than antigen presenting cells (APCs), such as epithelial cells[1]. Some studies also suggest that CD74 might be expressed independently of class Ⅱ MHC, indicating additional functions[2]. Various studies have indicated that CD74 is highly expressed in inflammatory disorders and cancers. It also acts as a receptor for macrophage migration inhibitory factor (MIF) and facilitates adhesion of *Helicobacter pylori* (*H pylori*) to gastric epithelial cells (GECs)[3,4]. CD74 expression is increased during *H pylori* infection, chronic inflammatory conditions of the gastrointestinal (GI) tract, and gastric and colon cancers. One critical function it has in carcinogenesis is to act as an accessory signaling molecule for cell proliferation. This molecule is particularly important in the complex immunological mechanisms of the gastrointestinal tract and in the link between chronic inflammation and carcinogenesis in the GI tract.

THE ROLE OF CD74 IN ANTIGEN PRESENTATION

CD74 was initially characterized for its role in regulating class Ⅱ MHC folding and intracellular sorting and has been studied in most detail in APCs. Newly synthesized CD74 self-assembles as a trimer and this trimer serves as a scaffold onto which class Ⅱ MHC molecules assemble. CD74 blocks the peptide binding cleft of class Ⅱ MHC and prevents premature binding of antigenic peptides. Upon endocytosis of antigens, CD74 directs the class Ⅱ MHC complex to the endosomal pathway using two di-leucine-based signals[5]. Within an endosomal compartment CD74 is digested, leaving just one residual peptide, CLIP (amino acids 91-99), blocking the peptide binding cleft of class Ⅱ MHC. A class Ⅱ MHC-like molecule, HLA-DM, then binds to the class Ⅱ MHC and CLIP is released leaving the peptide binding cleft open for antigenic peptide binding. Class Ⅱ MHC molecules with bound peptides are then exported to the surface of the

antigen presenting cell (APC) for presentation of foreign peptides to T cells. CD74 plays a crucial role in antigen presentation, as class Ⅱ MHC processing and regulation cannot properly occur in the absence of CD74.

In addition to APCs, other cells of the gastrointestinal tract, such as epithelial cells, express class Ⅱ MHC proteins and CD74 and act as APCs, which is an unusual trait of the GI tract. We have previously shown that gastric epithelial cells express class Ⅱ MHC proteins and are capable of processing antigens for presentation to T cells[6,7]. Expression of class Ⅱ MHC and the potential for antigen presentation have also been shown in intestinal epithelial cells. In one elaborate study by Hershberg *et al*, the trafficking of these molecules in epithelial cells was followed in a polarized manner outlining a functional system for antigen presentation[8]. Another important group of cells that express class Ⅱ MHC proteins are the subepithelial intestinal myofibroblasts (IMF)[9,10]. These cells are α-smooth actin positive stromal cells that exist in the lamina propria of the gut[11]. They also act as antigen presenting cells and play an important role in inflammatory diseases and carcinogenesis by releasing cytokines and growth factors and interacting with the immune cells of the lamina propria.

In conventional APCs, CD74 surface expression is low as CD74 is proteolytically removed in endosomes, as we and others[12,13] have shown. However, gastric epithelial cells express readily detectable levels of CD74 on the surface. When we examined human gastric biopsy sections by immunohistochemistry for epithelial expression of CD74, gastric biopsy samples from 44 random patients stained with anti-CD74 monoclonal antibodies (mAb) showed the expression of CD74 in the epithelium. In the samples that were positive for *H pylori* or had gastritis, CD74 staining was heavily increased[14]. This was corroborated by confocal microscopy studies of gastric epithelial cells grown as a polarized monolayer where expression was higher on the apical side of the cells[1].

CD74 ISOFORMS

CD74 is post-translationally glycosylated and exists in different isoforms. As evidence for this, our previous studies showed proteins with different mobilities when immunoprecipitated and subjected to gel electrophoresis[1]. After chemical deglycosylation, only the isoforms that result from alternative translation initiation or splicing were observed. The most common isoform is 33 kDa (p33), but p35, p41 and p43 isoforms also exist[15]. The p35 isoform contains a longer cytoplasmic tail due to the use of an alternative translation initiation site, while the p41 isoform results from alternative splicing, and p43 has both. Both the p33 and p35 isoforms appear to function in regulating class Ⅱ MHC antigen presentation. However, the p41 isoform might also play an important role in T cell selection in the thymus[16]. An important posttranslational modification of CD74 commonly seen is the addition of a glycosaminoglycan side chain, chondroitin sulfate. This isoform has been reported to act as an accessory protein during T cell activation through interaction with CD44 on T cells[17].

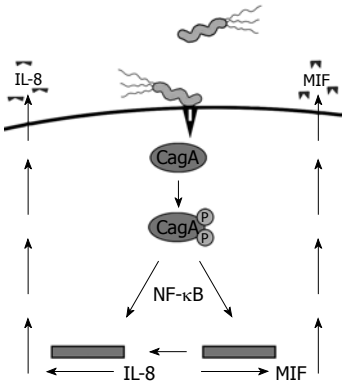

Figure 1 ***H pylori*** **induces MIF and IL-8 production by injecting CagA into GECs** ***via*** **a type Ⅳ secretion system.**

CD74 AS A RECEPTOR

CD74 has begun to emerge as a more versatile molecule beyond its well-known function of regulating class Ⅱ MHC trafficking. Multiple studies have revealed that cell surface expression of CD74 is not always dependent on class Ⅱ MHC[2,18]. This was found to be true in studies of colorectal mucosa and different types of lymphocytes by immunohistochemistry, immunoprecipitation, and a mutant cell line that did not express class Ⅱ MHC products. The expression of cell surface CD74 in the absence of class Ⅱ MHC suggests alternative functions for CD74 apart from antigen presentation.

CD74 as a cytokine receptor

Recently, CD74 has emerged as an integral component of a receptor complex for macrophage MIF[3]. MIF is a versatile cytokine-like molecule that mediates both innate and adaptive immunity, plays a role in chronic inflammation, and has also been linked to carcinogenesis. MIF is expressed by the GI tract during inflammatory conditions, *H pylori* infection, and cancers, suggesting the importance of this interaction[19-21]. *H pylori* utilizes a type Ⅳ secretion system to inject the CagA protein into GECs, which we have shown induces MIF production[19] (Figure 1). CagA has also been shown to induce NF-κB activation and IL-8 production[22]. This protein is not only important in inflammation by increasing proinflammatory cytokine production, such as IL-8, but is also associated with gastric cancer[23]. The current model of this receptor complex suggests that CD44 is required in order for MIF to induce signaling events[24]. This model might require the chondroitin sulfate modified isoform of CD74, since CD44 has been shown to bind only to this isoform thus far.

Another study has suggested that CD74 complexes with CXCR2, an interleukin-8 (IL-8) receptor, which is commonly expressed on macrophages and functions to recruit leukocytes to sites of infection[25]. CXCR2 is also expressed by the gastric epithelium[26]. Since gastric epithelial cells are central players in the inflammatory response, IL-8 may act via the gastric epithelium in various processes associated with gastric inflammation linked

to *H pylori* infection. The role of CXCR2 on the CD74 receptor complex has only recently been suggested and should be further investigated.

CD74 as a bacterial receptor

CD74 is an interesting example of a host cell receptor usurped by a pathogen because *H pylori* uses it to adhere to gastric epithelial cells (GECs). *H pylori* is a gram-negative spiral bacterium that colonizes the human gastroduodenal mucosa. Infection with *H pylori* usually begins in childhood and persists for decades if untreated. *H pylori* is recognized as a major contributor to chronic gastritis and peptic ulcer formation and is strongly associated with gastric carcinoma and lymphoma[27,28]. Due to the strength of the evidence supporting an association between adenocarcinomas of the gastric mucosa and *H pylori* infection, *H pylori* was classified as a class I carcinogen by the International Agency for Research on Cancer in affiliation with the World Health Organization[29]. Gastric cancer remains among the most common forms of cancer and is the second deadliest cancer worldwide. Gastric cancer accounts for approximately 700 000 deaths annually worldwide and in the US there are 24 000 new cases and 14 000 deaths annually[30]. The prevalence rates of *H pylori* seropositivity and the incidence of gastric cancer are highly associated within several populations from various countries. For instance, seropositivity can be as high as 80%-100% in some age groups in some countries or in minorities with lower incomes in the United States[31]. These groups have the highest risk of developing gastric cancer and/or gastric ulcers. Thus, *H pylori*-associated diseases represent a significant global and domestic problem and result in considerable morbidity, mortality, and societal costs.

H pylori adhesion to the gastric epithelium is important in successful colonization of the gastric mucosa. Adherent strains survive in the gastric mucosa, reach high bacterial densities, and can re-colonize, whereas non-adherent strains are cleared[32]. These observations support the notion that adhesion is essential in *H pylori* persistence and disease induction. An assortment of molecules on epithelial cells have been proposed as receptors for *H pylori* adherence including carbohydrate moieties [such as Lewis^b (Le^b) blood group antigen and sialyl-dimeric-Lewis^x (Le^x)], lysophospholipid, and other structures[33-35]. Our studies have indicated that *H pylori* also utilizes CD74 to attach to gastric epithelial cells (GEC)[4]. The binding of *H pylori* to CD74 on gastric epithelial cells was confirmed by a series of independent approaches. For instance, blocking of CD74 with antibodies significantly reduced the binding of *H pylori* to gastric epithelial cells. Immunoprecipitation revealed that *H pylori* predominantly binds to the 33 kDa isoform of CD74, but further investigation is needed to test for attachment to the CS modified isoform. As *H pylori* has been reported to bind to various glycoconjugates, including glycosaminoglycans[36], this isoform of CD74 might contribute to the overall interaction of *H pylori* with the host gastric epithelium. We also revealed that urease is the protein on *H pylori* that binds to CD74[37]. This interaction is particularly interesting because many bacteria express urease, so the possibility exists that there might be wider applications of this type of interaction with CD74 depending on urease sequence variation between bacteria.

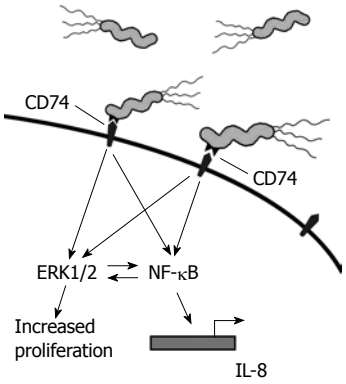

Figure 2 *H pylori* binds to CD74 on gastric epithelial cells and induces Nf-κB and Erk1/2 activation and IL-8 production.

After adhesion of *H pylori* to GEC, the expression of cell surface CD74 is increased[14]. We further showed that CD74 expression increases in gastric epithelial cells of infected humans and a recent study confirmed that this increase in CD74 expression also occurred in a mouse model of *H pylori* infection[38]. Upon *H pylori* binding to CD74, NF-κB activation occurs resulting in the production of proinflammatory cytokines, including IL-8. IL-8 plays a major role in the proinflammatory immune response to *H pylori* infection, therefore, the interaction of *H pylori* with the gastric epithelial cells might be of critical importance in the immune response to infection.

THE ROLE OF CD74 IN SIGNALING EVENTS

The role of CD74 in signal transduction was initially hypothesized when it was found to be phosphorylated and associated with proteins that coordinate various signal transduction pathways[39-41]. Interestingly, the observation that CD74 deficient mice have a defect in B cell development that results in decreased levels of follicular B cells provided insights into the important role of signals delivered through CD74 in B cell development. The cytosolic domain of CD74 alone was noted to induce B cell maturation by activation of NF-κB[42]. CD74 appears to promote B cell survival; therefore, it has been implicated in B cell neoplasms such as gastric mucosa-associated lymphoid tissue (MALT) lymphomas associated with *H pylori* infection.

Our studies have shown that the interaction of *H pylori* with CD74 on GECs induces NF-κB activation and IL-8 production, as shown in Figure 2. MIF has also been shown to bind to CD74[3]. One study outlines a role for CD44 in CD74 signaling. CD44 was required to initiate ERK1 and ERK2 signaling after MIF binding to CD74. Cells deficient in CD44 or transfected with truncated CD44 were not able to induce ERK signaling[24]. In B cells, MIF binding to CD74 led to AKT, Syk, and NF-κB activation, and proliferation in a CD44-dependant manner[43]. CXCR2 has also been shown to complex with

CD74 on monocytes[25]. This study also illustrated that MIF bound directly to CXCR2 and induced monocyte arrest. This study further showed that MIF might interact with CXCR4 on T cells and induce effector T cell arrest. However, it is not yet clear how CD44 is involved in the complexing of CD74 with CXCR2 and what signaling may be induced through CXCR4 since CXCR2 and CXCR4 are G protein-coupled receptors.

Other studies suggest that MIF signaling may also occur by non-receptor mediated endocytosis in addition to the above described receptors[44]. In this proposed mechanism, endocytosed or endogenous MIF interacts with the Jun activation domain-binding protein (Jab1), which is a transcriptional activator for AP-1[45]. Activation of AP-1 might affect cell cycle events by inducing degradation of the cyclin dependent kinase inhibitor, which is a tumor suppresser gene.

THE ROLE OF CD74 IN GI INFLAMMATION

CD74 is highly expressed in inflammatory disorders. We have shown it to be expressed on the gastric epithelium and up-regulated during *H pylori* infection[14]. Others have shown expression to be increased in ulcerative colitis, where overexpression was shown in DNA microarray profiles[46]. Additionally, CD74 is increased during inflammation associated cancers, such as gastric and colon[47,48]. Concurrently, MIF is highly expressed during many inflammatory conditions of the GI tract. We and others have shown that production is increased during *H pylori* infection[19,49]. MIF is also highly expressed during inflammatory bowel disorders (IBD), such as ulcerative colitis and Crohn's disease, where it is induced by intestinal bacteria[50]. In one study, elevated MIF levels were found in Crohn's disease patients at approximately six times higher levels than in healthy controls. Crohn's disease is an inflammatory bowel disorder where the immune system attacks part of the GI tract, and is accompanied by chronic inflammation. Also, this group went on to study MIF in murine colitis where they found colitis to be dependent on continuous MIF production, as evidenced by the protection from colitis by MIF-deficient mice or blocking MIF with monoclonal antibodies in mice with established colitis leading to reduced inflammation.

MIF or *H pylori* binding to CD74 induces NF-κB and subsequent cellular responses, such as the secretion of proinflammatory cytokines. MIF also increases inflammatory responses by overriding glucocortocoid suppression of inflammatory immune responses, including cytokines such as IL-1, IL-6, IL-8, and TNF-α[51]. One of the major proinflammatory cytokines produced after engagement of CD74 and receptor complexing is IL-8. IL-8 is a chemoattractant for neutrophils to a site of inflammation or infection. Upon arrival, they endocytose the antigen and form a phagosome in which reactive oxygen species and hydrolytic enzymes are released. While this process is crucial in fighting infections, it might also exacerbate inflammation in *H pylori* infection and inflammatory bowel disease[52-54]. In another study of glucocortocoid resistant ulcerative colitis, MIF was found to increase IL-8 production through the p38 MAPK pathway with isolated lamina propria mononuclear cells from patient biopsies[55]. When MIF was blocked with monoclonal antibodies after prednisolone treatment, activation of the p38 pathway and IL-8 decreased.

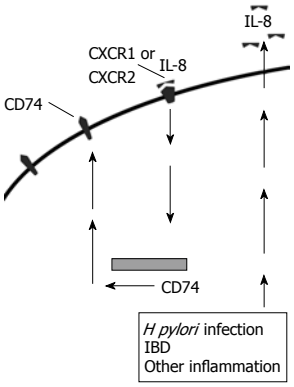

Figure 3 IL-8 produced by epithelial cells binds to CXCR1 and CXCR2 and up-regulates CD74 expression in an autocrine manner.

MIF might also contribute to inflammation by regulating Toll Like Receptor 4 (TLR4) expression on immune cells. TLR4 engagement by ligands such as bacterial LPS leads to proinflammatory cytokine production. This mechanism might be especially important in IBD, in which intestinal bacteria are a major contributor to the induction of the strong inflammatory response. In an *in vivo* study in mice, TLR4 expression in colonic tissue was not seen in MIF knockout mice, although it was in wild-type mice[56]. When the MIF knockout mice were administered rMIF, TLR4 expression was restored and further increased in colonic mice. In a human study of macrophages, neutralizing MIF or deleting the MIF gene resulted in decreased expression of TLR4 and a decreased response to LPS and gram negative bacteria; in broader experiments a decreased response to staphylococcal toxic shock and septic shock was demonstrated[57]. In addition, these cells did not respond well to LPS or gram negative bacteria and had a decreased expression of TLR4. The role of this receptor in the inflammation seen during *H pylori* infection is not clear because although *H pylori* LPS has been suggested to induce only weak responses, there are some studies suggesting it might contribute to the overall immune response to *H pylori*[58,59].

In addition to the role IL-8 plays in inflammation, we have previously shown that IL-8 increases expression of CD74 by gastric epithelial cells, both at the cell surface and mRNA levels[14] (Figure 3). Similarly, we found increased CD74 expression *in vivo*. Most of the *H pylori* infected samples and the samples with gastritis for reasons other than *H pylori* infection had much higher expression of CD74 than uninfected samples not exhibiting signs of gastritis. Other studies have further shown the expression of CD74 increased in ulcerative colitis and Crohn's Disease[46]. Increased CD74 expression could then go on to intensify inflammation by providing more free receptors for MIF or *H pylori* attachment.

THE ROLE OF CD74 IN GI CANCERS

CD74 has a strong link to carcinogenesis, as does MIF.

This ligand/receptor combination might be an important link between chronic inflammation and carcinogenesis. CD74 expression and MIF production have been shown to be increased during *H pylori* infection and gastric cancer[14,47]. High expression has also been noted in colon cancer along with highly increased serum concentrations of MIF in patients with colorectal cancers[60,61]. The contribution of CD74 to carcinogenesis is multifaceted. High levels of CD74 expression associated with class Ⅱ MHC expression might prevent tumor antigen presentation by blocking the peptide binding cleft and preventing antigenic peptide binding for presentation to T cells, rendering tumors less immunogenic. One study suggested this to be the case with colon neoplasms where expression was even increased from low to high grade neoplasms[48]. Chronic inflammation and IL-8 production leads to a prolonged increase in CD74 expression, which might not only decrease antigen presentation, but also exacerbate IL-8 production upon engagement by MIF or *H pylori*. MIF binding to the CD74 receptor complex also promotes proliferation of epithelial cells[19,62]. Long term increased proliferation overtakes natural cell cycle events and sets the stage for carcinogenesis.

MIF binding to CD74 might contribute to carcinogenesis in chronic conditions through the up-regulation of proinflammatory cytokines, including IL-8, which up-regulates CD74 and has its own mechanisms leading to increased proliferation, tumor growth, and angiogenesis. MIF or IL-8 binding to their receptors on epithelial cell surfaces induces the shedding of EGFR ligands in a mellatoprotease-dependent manner, and activation of EGFR through engagement of these ligands[63]. We and others have shown that this pathway is activated during *H pylori* infection[62,64,65]. Additionally, we found that EGFR expression is up-regulated in gastric epithelial cells during *H pylori* infection by MIF and IL-8" after the word infection. The EGFR is highly expressed in various cancers and is involved in pro-inflammatory responses and pro-carcinogenic events, including cell proliferation, migration, and invasion. Expression and activation of this receptor is well-documented in gastric and intestinal cancers[66,67]. One study suggests a correlation between EGFR expression on tumor cells, proliferation, and prognosis in gastric cancer[68]. Another study showed that treatment with antisense RNA for EGFR inhibited gastric tumor growth in a mouse model[69]. Blocking EGFR or its ligands is being studied in order to develop more effective treatments for GI cancers.

MIF also increases epithelial cell proliferation after binding to CD74. One way MIF might affect proliferation and cell cycle events is by regulating p53 tumor suppressor. Numerous cell cycle and apoptosis genes are controlled by p53. We have shown that phosphorylation of the p53 is decreased after MIF binding to CD74[19]. Others have shown that MIF can interact directly with p53 and prevent translocation to the nucleus where it becomes activated and acts as a transcription factor for apoptotic genes[70]. When p53 is blocked from transport to the nucleus, apoptotic pathways are decreased and proliferation increases. Suppression of p53 in macrophages results in a more robust inflammatory response, implying a further link between p53 and inflammation[71].

CONCLUSION

CD74 is a much more versatile molecule than originally thought, playing many important roles in the immune system. Of crucial importance is the role it plays in class Ⅱ MHC processing and the regulation of antigen presentation. This is important in the GI tract because epithelial cells and subepithelial myofibroblasts express CD74 and act as antigen presenting cells. Furthermore, the expression of CD74 on the cell surface might increase chronic inflammatory responses important in both *H pylori* infection and IBD. As a receptor for MIF, CD74 also plays and crucial role in chronic inflammation and might represent a major link between chronic inflammation and carcinogenesis in gastric and intestinal cancers. Development of therapeutics for cancer involving blocking CD74 might provide effective treatments for GI cancers.

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ORIGINAL ARTICLES

Protective effect of *Radix Astragali* injection on immune organs of rats with obstructive jaundice and its mechanism

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Abstract

AIM: To observe the protective effect of *Radix Astragali* injection on immune organs (lymph nodes, spleen and thymus) of rats with obstructive jaundice (OJ) and its mechanism.

METHODS: SD rats were randomly divided into sham-operation group, model control group and *Radix Astragali* treatment group. On days 7, 14, 21 and 28 after operation, mortality rate of rats, pathological changes in immune organs, expression levels of Bax and nuclear factor (NF)-κB p65 proteins, apoptosis indexes and serum tumor necrosis factor (TNF)-α level in spleen and thymus were observed, respectively.

RESULTS: Compared to model control group, the number of dead OJ rats in *Radix Astragali* treatment group decreased ($P > 0.05$). The TNF-α level (27.62 ± 12.61 *vs* 29.55 ± 18.02, 24.61 ± 9.09 *vs* 31.52 ± 10.95) on days 7 and 21, the pathological severity score for spleen [0.0 (0.0) *vs* 0.0 (2.0) on days 7 and 14 and for lymph nodes [0.0 (1.0) *vs* 1.0 (2.0), 1.0 (0.0) *vs* 2.0 (1.0)] on days 21 and 28, the product staining intensity and positive rate of Bax protein in spleen [0.0 (0.0) *vs* 1.0 (2.0), 0.0 (1.0) *vs* 2.0 (1.5) and thymus [0.0 (0.0) *vs* 1.0 (2.0), 0.0 (1.0) *vs* 2.0 (1.5)] on days 14 and 28, the apoptotic indexes [0.0 (0.0) *vs* 0.0 (0.01)] in spleen and thymus [0.0 (0.0) *vs* 0.0 (0.01) on days 14 and 21 were significantly lower in *Radix Astragali* treatment group than in model control group ($P < 0.05$).

CONCLUSION: *Radix Astragali* has protective effects on immune organs of OJ rats by relieving the pathological changes in immune organs, reducing TNF-α level and inhibiting Bax expression and apoptosis in spleen and thymus.

Key words: *Radix Astragali*; Traditional Chinese medicine; Obstructive jaundice; Rat; Immune organ; Tumor necrosis factor-α; Bax; Nuclear factor-κB; Apoptosis; Tissue microarry

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INTRODUCTION

Obstructive jaundice (OJ) is a kind of common clinical manifestation. The pathogenesis and treatment of OJ have been a hot topic in medical field for a long time[1-3]. Since

systemic inflammatory response syndrome and multiple organ dysfunction syndrome were studied in recent years, immune function impairment concomitant with OJ has gradually attracted wide attention and is considered as a cause of death in OJ patients[4-7]. Therefore, one of the important approaches to treatment of OJ is to restore the functions of immune organs[8-10].

Development and utilization of traditional Chinese medicine have good prospects in therapy for OJ since it has a lower cost, more extensive pharmacological effects and fewer side effects. *Radix Astragali,* dried root of *Astragalus membranaceus*, is sweet in taste with a warm property and mainly produced in Inner Mongolia Autonomous Region, Shanxi, Gansu and Heilongjiang Provinces of China. The raw *Radix Astragali* can be used to consolidate the exterior of body. *Radix Astragali* can regulate sweating, warm muscles, strengthen striae, invigorate Qi (vital energy), and alleviate heat in muscles due to Qi deficiency. *Radix Astragali* can also be used in treatment of chicken pox or other diseases with vesicular-papules due to inadequate "dispersing of the evils". *Radix Astragali* invigorates Qi, ascends the Yang-Qi, protects Qi and consolidates the exterior of body, promotes diuresis, relieves edema (generalized swelling of the body), supports Qi to promote skin wound/ulcer healing and tissue/muscle regeneration. *Astragalus* injection is made of extraction from *Radix Astragali*. Since *Astragalus* injection contains polysaccharide, saponin, flavone and trace elements, it has a variety of pharmacological effects and increases the immunity and protects the liver and kidneys[11-15]. It has been shown that cellular immune function decreases in OJ rats[16], which can be successfully treated with *Astragalus* injection.

At present, studies about the effects of *Astragalus* injection on immune organs during OJ are not available. This study was to investigate the protective effect of *Astragalus* injection on immune organs of OJ rats and its mechanism. The results may provide an experimental basis for its application in clinical practice.

MATERIALS AND METHODS

Materials

Healthy male SD rats of clean grade, weighing 270-330 g, were provided by Laboratory Animal Research Center, Zhejiang University of Traditional Chinese Medicine (China). Sodium pentobarbital was purchased from Sigma Corporation (USA). *Radix Astragali* injection (10 mL vial contains active components equivalent to 20 g of the original medicine) was purchased from Chiatai Qingchunbao Pharmaceutical Co, Ltd (China). Serum tumor necrosis factor (TNF)-α ELISA kits were purchased from Shanghai Senxiong Technological Company (China). Anti-nuclear factor (NF)-κB P65 and anti-Bax antibodies were purchased from Santa Cruz Biotechnology, Inc (USA). TUNEL assay kits were purchased from Takara Bio Inc (Jingdu, Japan).

Animal grouping and preparation of OJ models

One hundred and eighty OJ rats, enrolled in this study, were randomly divided into sham-operation group, model control group, and treatment group (n = 60), which were further subdivided into 7, 14, 21 and 28 d groups (n = 15) according to the time after operation. After the rats were anesthetized with intraperitoneal injection of 2.5% sodium pentobarbital (0.2 mL/100 g), their abdominal cavity was opened to identify and dissociate the common bile duct along the hepatoduodenal ligament. The proximal end of the common bile duct of rats in the model control and treatment groups was double-ligated with surgical threads, the common bile duct was cut off, and a layered suture of the abdominal wall was performed to close the abdominal cavity. The common bile duct of rats in the sham-operation group was dissociated but not ligated, and a layered suture of the abdominal wall was also performed to close the abdominal cavity. Rats in the treatment group received intraperitoneal *Radix Astragali* injection at a dose of 0.75 mL/100 g per day, while those in the sham-operation and model control groups received an equal volume of physiological saline solution until the end of 7-, 14-, 21- and 28-d observation periods in the corresponding groups.

Determination of experimental parameters

Mortality rates of rats in different groups were recorded. Rats were killed after anesthesia with sodium pentobarbital in batches, serum was collected to measure TNF-α level by ELISA, and pathological changes in immune organs (lymph nodes, spleen and thymus) were observed. Pathological severity of immune organs was scored according to related standards. Tissues of spleen, thymus and lymph nodes were cut into sections, but the sections of lymph node were not stained. Changes in expression levels of Bax and NF-κB P65 proteins, as well as apoptosis index of spleen and thymus were observed, respectively.

Immunohistochemical staining of Bax and NF-κB P65 proteins in intestinal mucosa

Envision two-step method was used to detect the expression levels of Bax and NF-κB P65 proteins in intestinal mucosa. The staining intensity was evaluated according to the extent of cell coloration: "-" represents negative staining; "+" represents mild staining with positively stained cells showing a yellow pigment; "++" represents moderate staining with positively stained cells showing a brown pigment; "+++" represents intense staining with positively stained cells showing a dark brown pigment; (-) indicates no positive cells; (+) indicates less than 25% of positive cells; (++) indicates 26%-50% of positive cells; and (+++) indicates over 50% of positive cells, each of which was scored as 0, 1, 2 and 3 points, respectively.

Detection of apoptotic index in intestinal mucosa with TUNEL staining

DNA nicking in tissue sections was observed with *in situ* end-labeling (TUNEL) staining. In brief, sections were

Table 1 Comparison of serum TNF-α levels in different groups (ng/L, mean ± SD)

Group	7 d	14 d	21 d	28 d
Sham-operation group	12.89 ± 1.63	12.25 ± 3.37	14.21 ± 3.24	15.61 ± 4.84
Model control group	29.55 ± 18.02[b]	34.10 ± 8.94[b]	31.52 ± 10.95[b]	57.66 ± 12.99[b]
Treatment group	27.62 ± 12.61[a,b]	27.20 ± 9.34[b]	24.61 ± 9.09[a,b]	39.01 ± 9.62[b]

[a]$P < 0.05$ *vs* model control group; [b]$P < 0.01$ *vs* sham-operation group.

baked at 60℃ for 30 min, deparaffinaged, and washed with Milli-Q for 5 min. Tissue was processed with protease K (10 μg/μL) at room temperature for 15 min and washed with phosphate-buffered saline (PBS) for 5 min. A 3% H_2O_2 solution was used to block endogenous peroxydase for 5 min, washed twice with PBS, 5 min each time. Thirty microliters of reaction solution at freezing condition (TdT enzyme : labeling safe buffer = 1:10) was added, incubated at 37℃ for 90 min, and washed twice with PBS, 5 min each time. Fifty microliters of anti-FITC HRP conjugate was added, incubated at 37℃ for 30 min, and washed twice with PBS, 5 min each time. DAB was colored, washed with Milli-Q, counterstained with hematoxylin, and washed with water after differentiation till it became blue. The DAB was routinely dehydrated and mounted onto neutral gum. Apoptotic index was calculated following the equation: Apoptotic index = apoptotic cell count/total cell count × 100%.

Statistical analysis

Data were input into the Excel sheet and read into SPSS 15.0 for further analysis. Normal data were expressed as mean ± SD while abnormal data were expressed as median (interquartile range). Analysis of variance and pairwise comparison were used for normal data, whereas abnormal data were subjected to non-parametric tests, of which Kruskal-Wallis H test was used for pairwise comparison and Mann-Whitney *U* test was used for multiple comparisons. Yates' χ^2 test was used for inter-group comparisons of mortality rates.

RESULTS

Comparison of mortality rates

All rats in the sham-operation group were alive after operation. Two rats in the model control group and one rat in the treatment group died on day 7 after operation. Four rats in the model control group and three rats in the treatment group died on day 14 after operation. Four rats in the model control group and four rats in the treatment group died on day 21 after operation. Seven rats in the model control group and six rats in the treatment group died on day 28 after operation. The total mortality rate of rats in the model control and treatment groups on day 28 was significantly higher than that of rats in the sham-operation group ($P < 0.001$). No significant difference was found in mortality rate between the model control and treatment groups.

Comparison of serum TNF-α levels

The serum TNF-α level was significantly lower in sham-operation group than in model control and treatment groups at different time points after operation ($P < 0.01$), and was significantly lower in treatment group than in model control group on days 7 and 21 after operation ($P < 0.05$, Table 1).

Pathological changes in spleen

In the sham-operation group, the morphology of spleen was normal with no gross pathological changes under light microscope.

In the model control group, the size of spleen increased by 1.2-1.5 folds and the texture of spleen became fragile with no change in color on day 7 after operation. The spleen became enlarged with a thickness of above 0.6 cm and a deeper color and its texture became fragile on day 14 after operation. The spleen was 4 cm × 1 cm × 1 cm in size and its texture became fragile with a purple black color on days 21 and 28 after operation. Under light microscope, the spleen of all rats was grossly normal on day 7 after operation. Fusion, enlargement or spotty necrosis of follicular germinal center in the white pulp of spleen, hyperplasia of fibrous tissue in sinus, and arteriolar sclerosis in spleen of most rats were observed on day 14 after operation. The spleen of few rats was grossly normal. Fusion, enlargement or spotty necrosis of follicular germinal centers in the white pulp of spleen, hyperplasia of fibrous tissue in sinus, and arteriolar sclerosis in spleen of few rats were seen on days 21 and 28 after operation. The spleen of some rats was grossly normal (Figure 1A).

In the treatment group, no significant difference was found in pathological changes at all time points after operation compared to the model control group. Under light microscope**,** no significant difference in pathological changes was noted at each time point after operation. The spleen of most rats was grossly normal. Arteriolar sclerosis in spleen of few rats was seen (Figure 1B).

The pathological scoring standards for spleen are listed in Table 2. The pathological scores were significantly lower for sham-operation group than for model control group on day 14 after operation ($P < 0.05$), and were significantly lower for treatment group than for model control group on days 7 and 14 after operation ($P < 0.05$, Table 3).

No significant difference was found in product staining intensity and in positive rate of NF-κB protein in spleens of all groups (Table 3).

The product staining intensity and positive rate of Bax protein were significantly lower in sham-operation and treatment groups than in model control group on day 28 after operation ($P < 0.05$, Table 3).

The apoptosis index was significantly lower in sham-

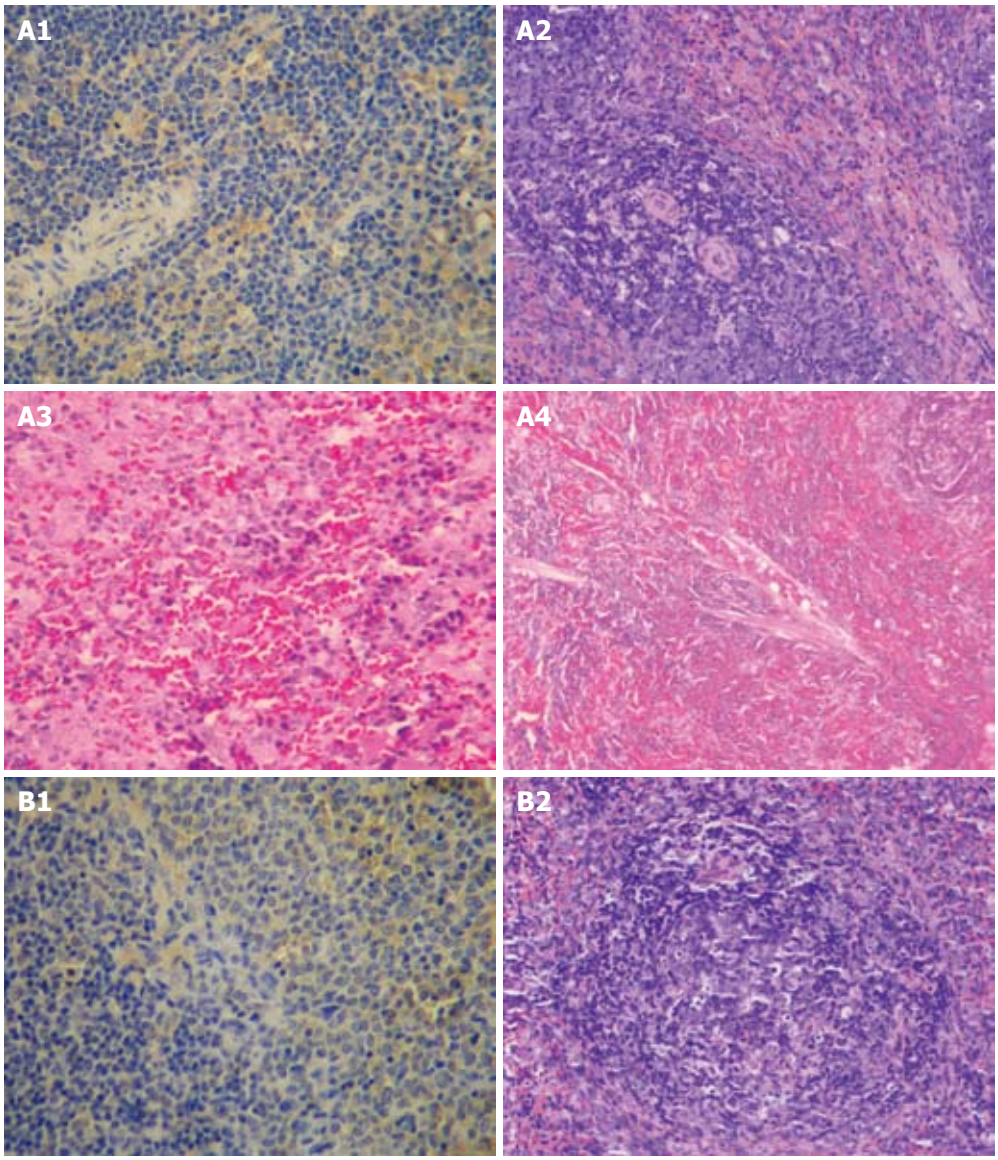

Figure 1 Pathological changes in spleen of model control group (A) and treatment group (B). A1: 21 d. Spleen (++) (Bax, × 200); A2: 28 d. Thickening of the wall of small spleen arteries as well as expansion and congestion of the red pulp (HE, × 200); A3: 28 d. Enlargement of spleen sinusoid, hyperplasia of cells in the spleen sinus as well as inflammatory cell infiltration and hemorrhage (HE, × 200); A4: 28 d. Enlargement of spleen sinusoid and hyperplasia of fibrous tissue (HE, × 100); B1: 21 d. Spleen (+) (Bax, × 200); B2: 28 d. Focal necrosis in spleen lymphoid follicles (HE, × 100).

Table 2 Pathological severity scoring standards for spleen

Score standards	Observation indexes
0	Normal
1	Necrosis of follicle center
2	Blood sinus dilation or arteriolar sclerosis
3	Necrosis of follicle center, blood sinus dilation and arteriolar sclerosis

operation group than in model control group on days 7, 14 and 28 after operation ($P < 0.05$), and was significantly lower in treatment group than in model control group on day 14 after operation ($P < 0.05$, Table 3).

Pathological changes in lymph nodes

In sham-operation group, the gross morphology of lymph nodes was normal. Under light microscope, no significant difference was observed in pathological changes at different time points after operation. The morphology and structure of lymph nodes were grossly normal. Enlargement of follicular germinal centers and hyperplasia of sinus cells were seen in few rats (Figure 2A).

Table 3 Comparison of different pathological indexes for spleen, M (Q_R)

Index	Time (d)	Sham-operation group	Model control group	Treatment group
Pathological severity score	7	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)[c]
	14	0.0 (1.0)[c]	0.0 (2.0)	0.0 (0.0)[c]
	21	0.0 (0.0)	1.0 (2.0)	0.0 (0.0)
	28	0.0 (0.0)	0.0 (1.0)	0.0 (0.0)
Product staining intensity and positive rate of Bax	7	0.0 (0.0)[c]	1.0 (2.0)	0.5 (1.0)[a]
	14	0.0 (1.0)	1.0 (2.0)	0.0 (0.0)[c]
	21	0.0 (0.0)[c]	1.0 (2.0)	0.0 (2.0)
	28	0.0 (0.0)[c]	2.0 (1.5)	0.0 (1.0)[c]
Apoptosis index	7	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
	14	0.0 (0.0)	0.0 (0.01)	0.0 (0.0)[c]
	21	0.0 (0.0)[c]	0.0 (0.01)	0.0 (0.0)
	28	0.0 (0.0)[c]	0.01 (0.02)	0.0 (0.0)
Product staining intensity and positive rate of NF-κB	7	0.0 (0.0)	0.0 (1.0)	0.0 (0.0)
	14	0.0 (1.0)	0.0 (2.0)	0.0 (0.0)
	21	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
	28	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)

[a]$P < 0.05$ *vs* sham-operation group; [c]$P < 0.05$ *vs* model control group.

In model control group, lymph nodes became yellow in 50% of the rats on day 7 after operation, but no

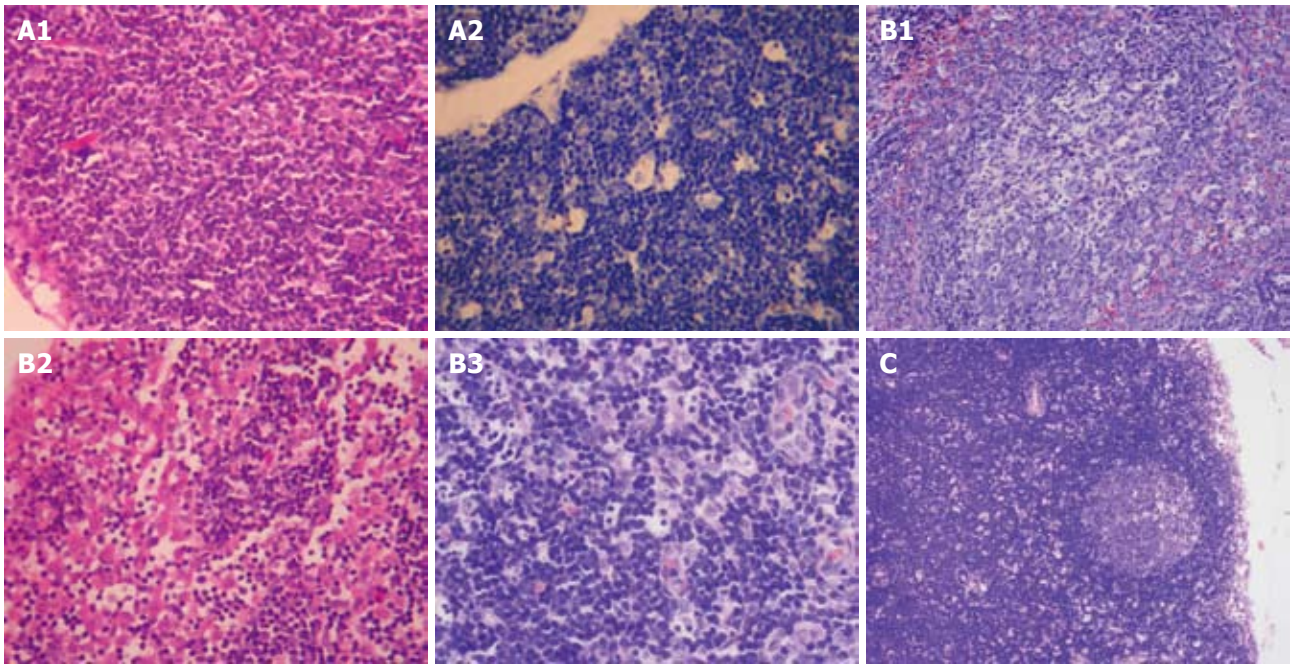

Figure 2 Pathological changes in lymph nodes of sham-operating group (A), model control group (B), and treatment group (C). A1: 28 d. Mainly normal lymph nodes (HE, × 200); A2: 28 d. No apoptotic cells in lymph node (TUNEL, × 200); B1: 21 d. Focal necrosis in lymphoid follicles and formation of germinal centers (HE, × 200); B2: 21 d. Expansion of lymph sinus, sinus cell hyperplasia and inflammatory cell infiltration (HE, × 200); B3: 28 d. Enlargement and spotty necrosis in germinal centers of lymph nodes, expansion of lymph sinus, hyperplasia of sinus cells and infiltration of neutrophils in lymph sinus (HE, × 200); C: 28 d. Clear follicular structure and fewer necrotic spots in lymph nodes (HE, × 100).

Table 4 Comparison of pathological severity scores for lymph nodes

Groups	7 d	14 d	21 d	28 d
Sham-operation group	0.0 (2.0)[c]	0.0 (1.0)[c]	0.0 (1.0)[c]	0.0 (1.0)
Model control group	1.0 (2.0)	1.0 (1.0)	1.0 (2.0)	2.0 (1.0)
Treatment group	0.0 (0.0)[a]	0.0 (1.0)	0.0 (1.0)[c]	1.0 (0.0)[c]

[a]$P < 0.05$ *vs* sham-operation group; [c]$P < 0.05$ *vs* model control group.

changes were found in the texture of lymph nodes at all time points after operation. Under light microscope, no significant difference in pathological changes was observed at all time points after operation. Enlargement of follicular germinal centers and hyperplasia of sinus cells were seen in most rats and few rats showed no obvious pathological changes in lymph nodes with spotty necrosis in the mantle zone and germinal centers on days 7, 14, 21 and 28 after operation (Figure 2B).

In the treatment group, no significant difference in pathological changes was observed at all time points after operation compared to the model control group. Under light microscope, no obvious difference was found in lymph node pathological changes at all time points after operation. In most rats, enlargement of lymph nodes in germinal centers and hyperplasia of cells in lymph sinus were observed with spotty necrosis of lymph nodes in the mantle zone and germinal centers of (Figure 2C).

The pathological scoring standards for lymph nodes have been described elsewhere[17]. The pathological score was significantly lower for sham-operation group than for model control group on days 7, 14 and 21 after operation ($P < 0.05$). The pathological score was significantly lower for sham-operation group than for treatment group on day 7 after operation ($P < 0.05$) and was significantly lower for treatment group than for model control group on days 21 and 28 after operation ($P < 0.05$, Table 4).

Pathological changes in thymus

In sham-operation group, no significant difference was found in thymus pathological changes at all time points after operation compared to model control group, and the thymus tissue of all rats was grossly normal (Figure 3A).

In model control group, the thymus of rats was mildly shrunken on day 7 after operation, moderately shrunken and jaundiced on day 14 after operation, and severely shrunken and jaundiced on days 21 and 28 after operation. Under light microscope, no significant difference was noted in thymus pathological changes at all time points after operation. The thymus tissue of most rats was grossly normal. An obscure boundary between thymus cortex and medulla was occasionally seen. The thymus tissue was grossly normal on day 14 after operation. The thymus pathological changes were similar on days 14, 21 and 28 after operation (Figure 3B).

In treatment group, no significant difference in thymus pathological changes was observed on days 7 and 14 after operation compared with model control group. The thymus became mildly jaundiced with no obvious shrinkage on days 21 and 28 after operation. Under light microscope, the thymus tissue of most rats was grossly

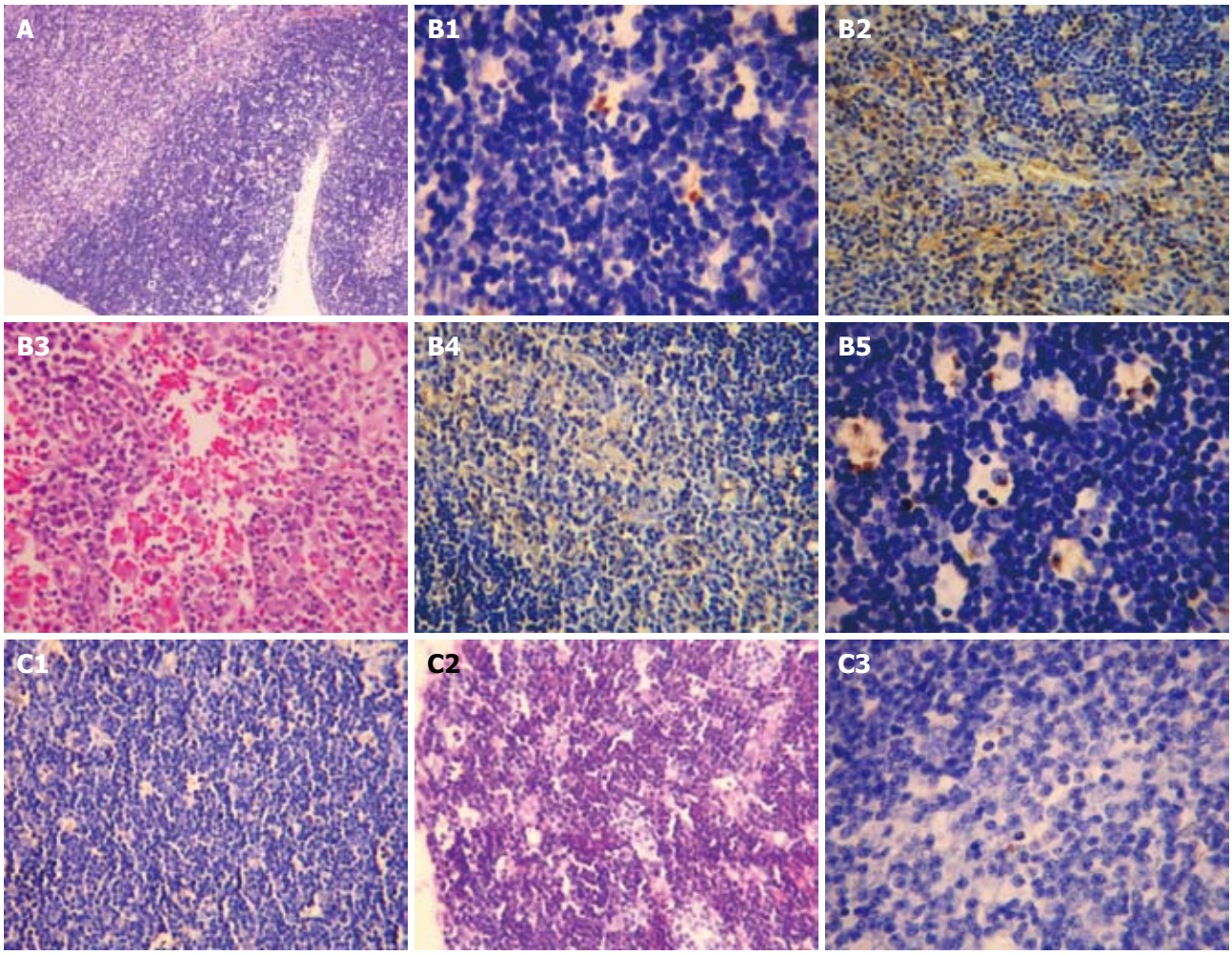

Figure 3 Pathological changes in thymus of sham-operated group (A), model control group (B), and treatment group (C). A1: 28 d. Clear structure of thymus lobules and a clear boundary between thymus cortex and medulla (HE, × 100); B1: 7 d. Sporadic apoptotic cells in thymus (TUNEL, × 400); B2: 21 d. Thymus (++) (NF-κB p65, × 200); B3: 28 d. An obscure boundary between the thymus cortex and medulla with hemorrhage in the medulla (HE, × 200); B4: 28 d. Thymus (++) (Bax, × 200); B5: 28 d. Sporadic apoptotic cells in thymus (TUNEL, × 400); C1: 21 d. Thymus (not shown) (NF-κB p65, × 200); C2: 28 d. Normal thymus (HE, × 200); C3: 28 d. Sporadic apoptotic cells in thymus (TUNEL, × 400).

Table 5 Comparison of different pathological indexes for thymus, *M* (Q_R)

Index	Time (d)	Sham-operation group	Model control group	Treatment group
Product staining intensity and positive rate of Bax	7	0.0 (0.0)[c]	1.0 (2.0)	0.5 (1.0)[a]
	14	0.0 (1.0)	1.0 (2.0)	0.0 (0.0)[c]
	21	0.0 (0.0)[c]	1.0 (2.0)	0.0 (2.0)
	28	0.0 (0.0)[c]	2.0 (1.5)	0.0 (1.0)[c]
Apoptosis index	7	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
	14	0.0 (0.0)	0.0 (0.01)	0.0 (0.0)[c]
	21	0.0 (0.0)[c]	0.0 (0.01)	0.0 (0.0)[c]
	28	0.0 (0.0)[c]	0.01 (0.02)	0.0 (0.0)
Product of the staining intensity and positive rate of NF-κB	7	0.0 (0.0)	0.0 (2.0)	0.0 (0.0)
	14	0.0 (4.0)	2.0 (4.0)	0.0 (2.0)
	21	0.0 (2.0)	0.0 (2.0)	0.0 (0.0)
	28	0.0 (1.0)	0.0 (3.0)	0.0 (2.0)

[a]$P < 0.05$ *vs* sham-operating group; [c]$P < 0.05$ *vs* model control group.

normal. An obscure boundary between thymus cortex and medulla was seen in few rats (Figure 3C).

The pathological scoring standards for thymus have been described elsewhere [18]. No significant difference was observed in pathological scores for different groups (Table 5).

No significant difference was found in product staining intensity and positive rate of NF-κB protein in thymus of different groups (Table 5).

The product staining intensity and positive rate of Bax protein were significantly lower in sham-operation group than in model control group on days 7, 21 and 28 after operation ($P < 0.05$), in sham-operation group than in treatment group on day 7 after operation ($P < 0.05$), and in treatment group than in model control group on days 14 and 28 after operation ($P < 0.05$).

The apoptosis index for thymus was significantly lower in sham-operation group than in model control group on days 21 and 28 after operation ($P < 0.05$), and in treatment group than in model control group on days 14 and 21 after operation ($P < 0.05$).

DISCUSSION

It has been shown that the incidence rate of endotoxemia in OJ patients is as high as 39.3%[19], which is mainly

due to insufficient intestinal bile salt that leads to excessive multiplication of intestinal bacteria and decreased inactivation of endotoxins. Since the function of reticuloendothelial system is inhibited, gut-derived endotoxins are not effectively eliminated. As a result, large amounts of endotoxin enter into the blood resulting in endotoxemia. Endotoxin is a main factor for immune function impairment during OJ since it can directly stimulate Kupffer cells to release inflammatory mediators, including oxygen free radicals, TNF-α, IL-6 and IL-8, and thereby aggravates the inflammatory response of body[19-24]. TNF-α is the most important factor for mediating toxic effects of endotoxin. Excessive release of TNF-α can induce multiple organ injuries. We speculate that immune function impairment results from the damage to immune organs. The results of this study show that the serum TNF-α level and pathological scores for spleen and lymph nodes were significantly lower in sham-operation and treatment groups than in model control group, suggesting that TNF-α is involved in OJ-induced damage to immune organs, and *Astragalus* injection can significantly lessen the toxic effects of TNF-α on and improve the pathological changes in immune organs. We think that *Astragalus* injection exerts, to a certain degree, its protective effects on immune organs by suppressing the production of TNF-α. Although no statistically significant difference was observed in pathological scores for thymus, the pathological changes in thymus of *Astragalus* treatment group showed varying degrees of improvement compared with model control group. In model control group, the thymus showed varying degrees of jaundice and atrophy at all time points after operation, and an obscure boundary between the thymus cortex and medulla in parts of thymus tissue under light microscope. In contrast, the thymus in treatment group became jaundiced with no atrophy on days 21 and 28 after operation, and the boundary between the thymus cortex and medulla was obscure in few parts of thymus tissue under light microscope, suggesting that *Astragalus* has protective effects on immune organs.

NF-κB p65, a protein that is extensively distributed in cytoplasm of many cells, can regulate gene transcription in nuclei. It is a member of Rel family of transcriptional regulatory proteins and is involved in gene expression regulation of many inflammatory factors. When the body is under stress, NF-κB p65 is activated and binds to specific κB gene sequences, and thereby promotes gene transcription and protein synthesis of pro-inflammatory molecules, causes strong expression of inflammatory cytokines such as TNF-α and IL-6mRNA, accelerates toxic effect on cells in multiple organs, eventually leading to multiple organ dysfunction. Based on the expression levels of NF-κB p65 protein in spleen and thymus, we speculate that *Astragalus* has no inhibitory effects on the expression of NF-κB p65 protein in spleen and thymus of OJ rats.

Apoptosis is a self-protective strategy employed by the body for removal of the destroyed cells through initiating programmed gene expression under certain pathophysiological conditions[25]. In contrast to cell necrosis, apoptosis is an active and spontaneous process and does not induce dramatic inflammatory reaction. However, apoptosis as a mode of cell loss can also induce functional impairment of immune organs. Bax, a soluble protein encoded by a recently discovered apoptosis-promoting gene, shares the same protein family as Bcl-2 and is able to promote cell apoptosis[26,27]. In this study, the expression level of Bax protein was higher in spleen and thymus of model group than in those of sham-operation group. As a result, the apoptosis index was increased and pathological injury was aggravated, suggesting that Bax protein is involved in physiological or pathological cellular apoptosis of spleen and thymus. After treatment with *Astragalus* injection, the pathological changes in immune organs were improved and the expression level of Bax protein in spleen and thymus, apoptosis index and pathological scores for spleen and thymus were significantly lower in treatment group than in model control group, indicating that *Astragalus* injection can down-regulate the expression of Bax protein, suppress cell apoptosis and exert protective effects on immune organs.

In summary, *Astragalus* injection can improve pathological changes in immune organs, reduce serum TNF-α level, down-regulate expression of Bax protein in spleen and thymus, and suppress cell apoptosis, thereby exerting its protective effects on immune organs of OJ rats. Since *Astragalus* has diverse pharmacological actions, low cost and few side effects, it has a better application prospect and economic value.

COMMENTS

Background

Obstructive jaundice (OJ) is a kind of common clinical manifestation. The pathogenesis and treatment of OJ have been a hot topic in medical field for a long time. As systemic inflammatory response syndrome and multiple organ dysfunction syndrome were studied in recent years, immune function impairment concomitant with OJ has gradually attracted wide attention and is considered as a cause of death in OJ patients. Therefore, one of the important approaches to treatment of OJ is to restore the functions of immune organs.

Research frontiers

Development and utilization of traditional Chinese medicine have good prospects in therapy for OJ since it has lower cost, more extensive pharmacological effects and fewer side effects. Since *Astragalus* injection contains polysaccharide, saponin, flavone and trace elements, it has a variety of pharmacological effects and plays an important role in increasing the immunity of body and protecting the liver and kidney. This study demonstrated that *Radix Astragali* could exert its protective effects on immune organs of OJ rats by relieving the pathological changes in immune organs, reducing tumor necrosis factor (TNF)-α level, and inhibiting Bax expression and apoptosis in spleen and thymus.

Innovations and breakthroughs

At present, no studies about the effects of *Astragalus* on immune organs during OJ are available. In the present study, we investigated the protective effect of *Astragalus* injection on immune organs of OJ rats and its mechanism, which may provide an experimental basis for its application in clinical practice.

Applications

Astragalus has diverse pharmacological actions, low cost and few side effects, and thus can be applied in clinical practice.

Terminology

Nuclear factor (NF)-κB p65, a protein that is extensively distributed in cytoplasm of many cells, is able to regulate gene transcription in nuclei. TNF-α

is a most important factor for mediating the toxic effects of endotoxins. Bax, a soluble protein encoded by a recently discovered apoptosis-promoting gene, shares the same protein family as Bcl-2 and is able to promote cell apoptosis.

Peer review

The manuscript describes the protective effects of *Radix astragali* injection on immune organs of rats with OJ. *Radix astragali* injection could reverse elevated TNF-α level, and spleen, thymus and lymph node lesions. Bax immunoreactivity and apoptosis could be observed after obstructive jaundice. This manuscript is largely descriptive by providing novel insights into the mechanism underlying the beneficial effect *of Radix astragali* on obstructive jaundice.

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ORIGINAL ARTICLES

Alisol B acetate induces apoptosis of SGC7901 cells *via* mitochondrial and phosphatidylinositol 3-kinases/Akt signaling pathways

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Abstract

AIM: To examine the effect of alisol B acetate on the growth of human gastric cancer cell line SGC7901 and its possible mechanism of action.

METHODS: The cytotoxic effect of alisol B acetate on SGC7901 cells was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Phase-contrast and electron microscopy were used to observe the morphological changes. Cell cycle and mitochondrial transmembrane potential ($\Delta\Psi m$) were determined by flow cytometry. Western blotting was used to detect the expression of apoptosis-regulated gene Bcl-2, Bax, Apaf-1, caspase-3, caspase-9, Akt, P-Akt and phosphatidylinositol 3-kinases (PI3K).

RESULTS: Alisol B acetate inhibited the proliferation of SGC7901 cell line in a time- and dose-dependent manner. PI staining showed that alisol B acetate can change the cell cycle distribution of SGC7901, increase the proportion of cells in G0-G1 phase and decrease the proportion of S phase cells and G2-M phase cells. Alisol B acetate at a concentration of 30 μmol/L induced apoptosis after 24, 48 and 72 h incubation, with occurrence rates of apoptotic cells of 4.36%, 14.42% and 21.16%, respectively. Phase-contrast and electron microscopy revealed that the nuclear fragmentation and chromosomal condensed, cells shrank and attachment loss appeared in the SGC7901 treated with alisol B acetate. Apoptosis of SGC7901 cells was associated with cell cycle arrest, caspase-3 and caspase-9 activation, loss of mitochondrial membrane potential and up-regulation of the ratio of Bax/Bcl-2 and inhibition of the PI3K/Akt.

CONCLUSION: Alisol B acetate exhibits an anti-proliferative effect in SGC7901 cells by inducing apoptosis. Apoptosis of SGC7901 cells involves mitochondria-caspase and PI3K/Akt dependent pathways.

Key words: Alisol B acetate; Apoptosis; Mitochondria; Phosphatidylinositol 3-kinases/Akt; SGC7901 cells

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INTRODUCTION

Gastric cancer is one of the most common malignancies in mankind and its incidence and mortality rank first in China[1]. Recent data indicate that the mortality of gastric cancer in China is tending to increase and it severely threatens the health and life of people[2]. At present, the management of gastric cancer mainly includes surgery and chemotherapy, but the curative effect of the existing chemotherapeutic drugs is not good enough and they have numerous side effects. Therefore, it has become a focus to search the drugs capable of preventing and treating gastric cancer and other malignancies.

Herbal medicines are an important source of novel agents with pharmaceutical potential. Alisol B acetate is a major ingredient isolated from *Alismatis rhizoma* and has been used for urological diseases in traditional Chinese medicine. In recent years, the pharmacological characterization of alisol B acetate has been identified and several biological activities have been defined, such

as the inhibitory effects on lipopolysaccharide[3], the inhibition of complementary activity[4,5] and antibody-mediated allergic reaction[6]. Furthermore, it has been demonstrated that alisol B acetate induces cell death in hepatoma and leukemia cells[7,8]. Later studies have shown that alisol B acetate induces Bax nuclear translocation and apoptosis in human hormone-resistant prostate cancer PC-3 cells[9].

However, no detailed data are available about the role and mechanisms of alisol B acetate in gastric carcinoma. In order to understand the role and mechanisms of alisol B acetate in the treatment of gastric carcinoma, we investigated the effect of alisol B acetate on the growth of human SGC7901 cells and the underlying intracellular signal transduction pathways involved in regulating apoptosis. We found that alisol B acetate-induced apoptosis is accompanied by the modulation of the Bcl-2 family, mitochondrial dysfunction and activation of caspases; in SGC7901 cells, alisol B acetate induced apoptosis *via* the mitochondrial death pathway; and the apoptosis induced by alisol B acetate was sensitized through inhibition of the PI3K/Akt signaling pathway.

MATERIALS AND METHODS

Materials

Gastric adenocarcinoma cell line (SGC7901) was obtained from our laboratory. Alisol B acetate was purchased from Wako Pure Chemical Industries (Osaka, Japan). Methyl thiazolyl tetrazolium (MTT), propidium iodide (PI), Tris-HCl, and Triton X-100 were obtained from Sigma Chemical Company (St. Louis, USA). Monoclonal antibodies to Bax, Bcl-2, Apaf-1 caspase-3 and caspase-9 were purchased from Santa Cruz Biotechnology Incorporation (Santa Cruz, CA, USA). PVDF membrane was obtained from Bio-Rad (CA, USA). Phospho-Akt (Thr308), Akt, PI3K antibodies were procured from Cell Signaling Technology (Beverly, MA, USA).

Cell culture and treatment with alisol B acetate

Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin at 37℃ humidified atmosphere containing 5% CO_2. The chemical compounds (alisol B acetate) were dissolved in dimethyl sulfoxide (DMSO), and diluted to appropriate concentrations with culture medium. The final concentration of DMSO in the culture medium did not exceed 0.1%.

MTT assay

Cells were plated at a density of 2×10^3/well in 96-well plates. Twenty-four hours later, cells were treated with alisol B acetate at different final concentrations from 10 to 80 μmol/L for 24 h or at 30 μmol/L concentration for the indicated time courses. Control cell cultures were treated with DMSO. After addition of test compounds, 20 μL MTT was added to each well. Four hours later, 100 μL of DMSO was added to each well after the medium was removed. Finally, absorbance was detected with an enzyme calibrator at 570 nm and cell viability= (A of study group/A of control group) × 100%. Experiments were done in triplicate. There were six wells for each concentration.

Morphological changes examined by phase-contrast and electron microscopy

Phase-contrast microscopic studies: SGC7901 cells were grown in 35 mm sterile petri plates and treated with various doses of alisol B acetate for 24 h. Morphological changes were observed under phase-contrast microscope.

Electron microscopy: Cells were cultured with alisol B acetate at a concentration of 30 μmol/L for 24, 48 and 72 h, then fixed with 2% paraformaldehyde/2% glutaraldehyde in 0.1 mol/L phosphate buffer (pH 7.4), followed by 1% osmium tetroxide. After dehydration, thin sections were stained with uranyl acetate and lead citrate for observation under a JEM 100 CX electron microscope (JEOL, Peabody, NY, USA).

Flow cytometric analysis of cell cycle

Cells were seeded in 6-well plates and treated with alisol B acetate at 30 μmol/L concentration for 24, 48 and 72 h. DMSO (0.1%)-treated cells served as control. After treatment, media were discarded. The adherent cells were washed with PBS, and 300 μL trypsin was added for 5 min at room temperature to detach the cells. Then, the cell suspension was centrifuged at 1500 r/min for 5 min at room temperature. Decanting of all the supernatant was followed by adding 1 mL of 70% methanol to the pellet. After incubation at 4℃ for at least 12 h, prior to the samples being analyzed by the flow cytometry (FCM) (Becton Dickinson), 1 mL of cold PI stain solution (20 g/mL PI, 20 g/mL RNase A, and 0.1% Triton X-100) was added to the mixture and it was incubated for 15 min in darkness at room temperature. The samples were analyzed by FCM (BD FACS CantoTM). The results were analyzed by Mod Fit LT 3.0 software.

Analysis of mitochondrial membrane potential

Changes of mitochondrial membrane potential were monitored by determination of the fluorescence of Rhodamine (Rh)-123. Cells were treated with or without alisol B acetate for the indicated time courses. At the end of treatment, the cells were finally harvested. Rh-123 was added to 1×10^6 cells in 5 mL complete growth medium to a final concentration of 5 g/L and cells were incubated at 37℃ in the dark for 30 min to allow Rh-123 uptake. Rh-123 loaded cells were washed with ice cold PBS and re-suspended in PBS. Changes in mitochondrial transmembrane potential as a result of mitochondrial perturbation were measured after staining with Rh-123[10]. Ten thousand events were examined by a FACSCAN flow cytometer and data were analyzed with the Macintosh Cell Quest software.

Western blotting analysis

After being treated with alisol B acetate for the indicated periods, the cells were washed with PBS and lysed in a buffer containing 20 mmol/L Tris-HCl, 150 mmol/L NaCl, 1% Triton X-100, 1.5 mmol/L $MgCl_2$, 1 mmol/L $NaVO_3$, 100 mmol/L NaF, 10% glycerol, 1mmol/L EGTA, 10 mmol/L sodium pyrophosphate, and 1 mmol/L phenylmethylsulfonyl fluoride, pH 7.5. Cell lysates were centrifuged at 12000 × *g* for 60 min at 4℃. The protein concentrations were determined using Bio-Rad protein assay (Bio-Rad Laboratories, USA). After SDS-PAGE, proteins were transferred to PVDF membranes for 2 h at 80 mA. The PVDF membrane was treated with TBST containing 50 g/L skimmed milk at room temperature for 2 h, followed by incubation with the first antibodies caspase-3, caspase-9, Apaf-1, Bcl-2, Bax, Akt, P-Akt, PI3K, respectively, at 4℃ overnight. After being washed with TBST for 30 min, the corresponding secondary antibody was added and incubated at room temperature for 1 h. The membrane was then washed three times for 15 min each with TBST and visualized with diaminobenzidine. Quantification of protein was detected with a Lumivision IMAGER (Aisin Seiki, Aichi). Each value represents the mean of triple experiments, and is presented as the relative density of protein bands normalized to β-actin.

Statistical analysis

Data were expressed as mean ± SD. Statistical correlation of data was checked for significance by ANOVA and Student's *t* test. Differences with $P < 0.05$ were considered significant. These analyses were performed using SPSS 11.0 software.

RESULTS

Alisol B acetate inhibited SGC7901 cell proliferation

To investigate the growth inhibition effects of alisol B acetate, the cells were treated with various concentrations of alisol B acetate for 24 h and 30 μmol/L for 8, 16, 24, 48 and 72 h. As shown in Figure 1A, cell viability was decreased remarkably after the cells were treated with 30, 50, 70 and 80 μmol/L alisol B acetate for 24 h. Only a minor inhibition of SGC7901 cell growth was observed in the presence of 20 μmol/L alisol B acetate. Growth was inhibited by more than 40% in cells exposed to 30 μmol/L alisol B acetate after 24, 48 and 72 h. Alisol B acetate had significant growth inhibitory effects on SGC7901 cells in a dose- and time-dependent manner. A concentration of 30 μmol/L alisol B acetate was used in all further experiments.

Alisol B acetate induces apoptosis

SGC7901 cells were treated with alisol B acetate (0, 30, 50 and 70 μmol/L) for 24 h, and phase-contrast microscopy revealed that some cells became round, blunt and smaller in size; light refraction was increased; and cells became detached and suspended in the medium, especially with 50 and 70 μmol/L alisol B acetate. In the control group, cells were regular in morphology and grew fully in patches and confluently, rarely sloughing off (Figure 2).

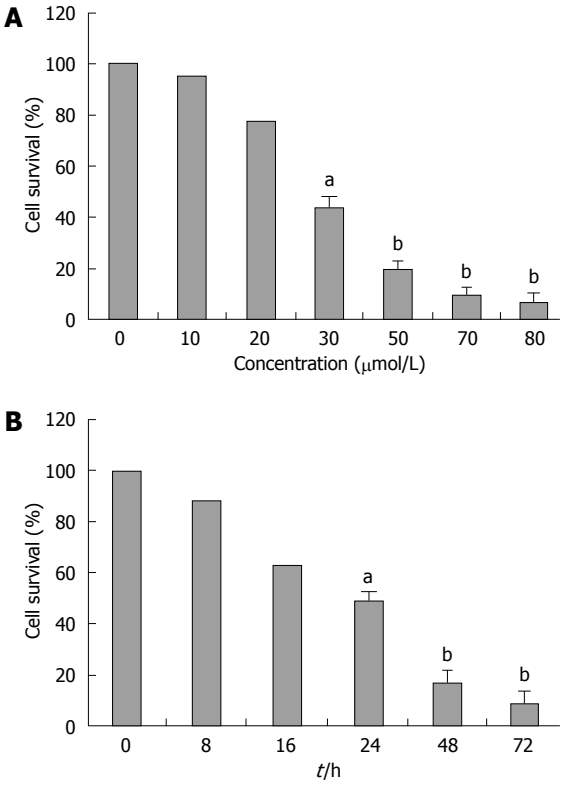

Figure 1 Effect of alisol B acetate on survival of SGC7901 cells. Cells were incubated in the absence or presence of various concentrations of alisol B acetate for 24 h (A), or at 30 μmol/L for different incubation times (B). $^aP < 0.05$, $^bP < 0.01$ *vs* control group (unpaired Student's *t* test).

The ultrastructural and morphological changes were also observed under electron microscope. As shown in Figure 3B and C, nuclear fragmentation, chromosome condensation and cell shrinkage were visible. Subsequent formation of apoptotic bodies were also observed (Figure 3D).

FCM with only PI staining showed (Figure 4) that treatment of SGC7901 cells with 30 μmol/L alisol B acetate for 72 h resulted in a higher number of cells in the G0/G1 phase (79.61%) compared with the control (40.46%). This increase was coupled with the decreased percentage of cells in S phase. After 72 h treatment, the percentage of S phase in alisol B acetate -treated cells was 16.55%, whereas 48.45% in the control cells. In addition, flow cytometric analysis also revealed the effect of alisol B acetate on the induction of apoptosis. As shown in Figure 4, the percentage of the sub/G1 fraction in alisol B acetate-treated cells was increased in a time-dependent manner, indicative of apoptotic cell death.

Alisol B-induced apoptosis is associated with mitochondrial pathways

Mitochondria played a major role in apoptosis triggered by many stimuli. The early loss of mitochondrial membrane potential is a hallmark of apoptosis[11]. We examined the effect of alisol B acetate on mitochondrial

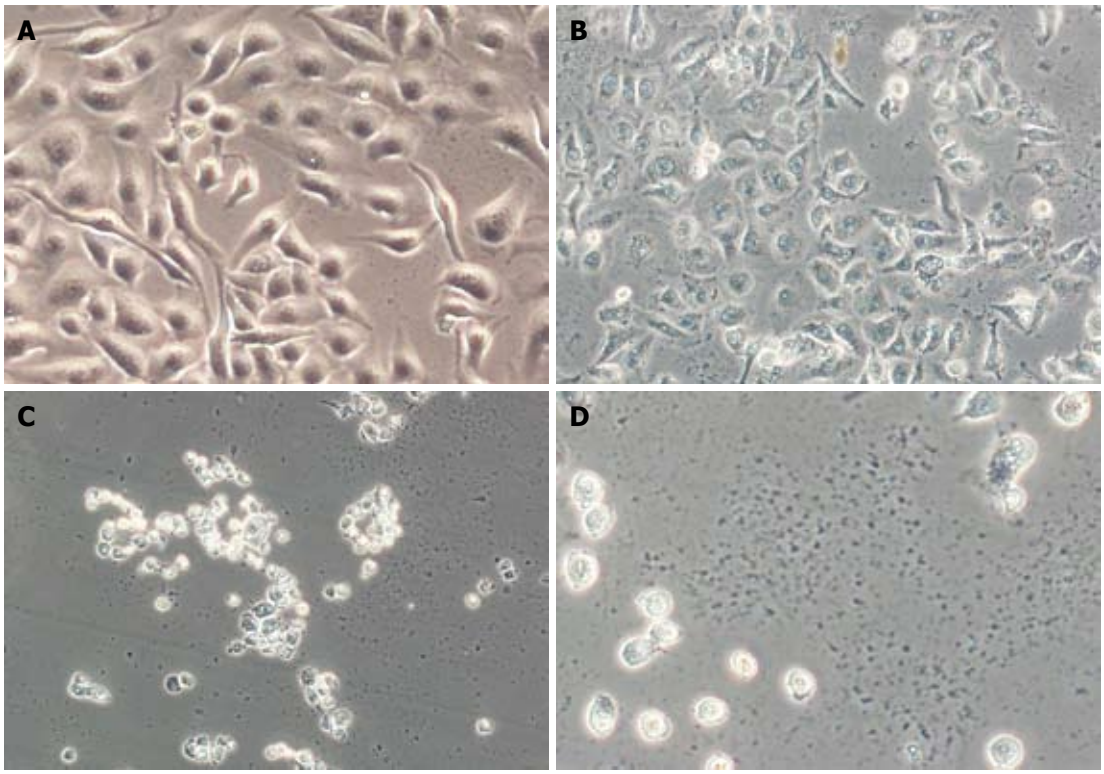

Figure 2 Morphology of SGC7901 cells exposed to alisol B acetate for different concentrations observed under phase-contrast microscope. A: Controls; B-D: SGC7901 cells were treated with 30, 50 and 70 μmol/L alisol B acetate for 24 h.

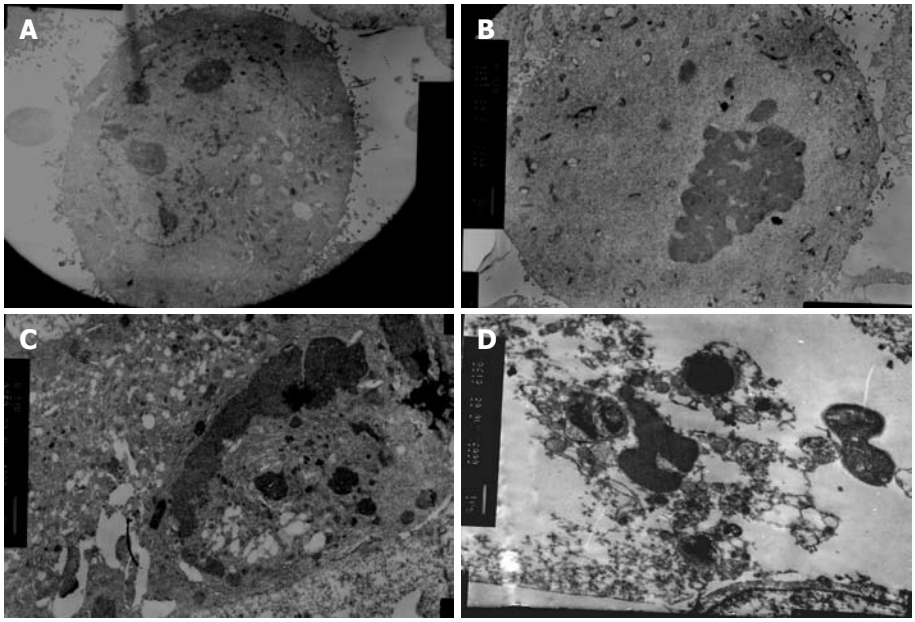

Figure 3 Morphological changes observed under electronic microscope. A: Control; B-D: Cells were incubated with 30 μmol/L alisol B acetate for 24, 48 and 72 h.

membrane potential by means of the potential sensitive Rh-123. After treatment of SGC7901 cells with 30 μmol/L alisol B acetate for 12, 24, 48 and 72 h, flow cytometric data revealed that disruption of mitochondrial membrane potential was 17.75%, 29.87%, 43.32% and 70.00%, respectively, while it was only 4.05% in the control group. The data demonstrated that alisol B acetate induced a time-dependent decrease in mitochondrial membrane potential, indicating the participation of mitochondria-related mechanism (Figure 5).

Up-regulation of Apaf-1 and Bax, activation of caspase-3 and caspase-9

To investigate the mechanism underlying apoptosis induced by alisol B acetate, we tested the effect of this compound on Bcl-2, Bax levels, two important regulators of apoptotic signaling pathways[12]. As shown in Figure 6, Western blotting analysis revealed that alisol B acetate -induced apoptosis did not alter Bcl-2 expression, but resulted in a time-dependent up-regulation of Bax expression, with a maximal up-

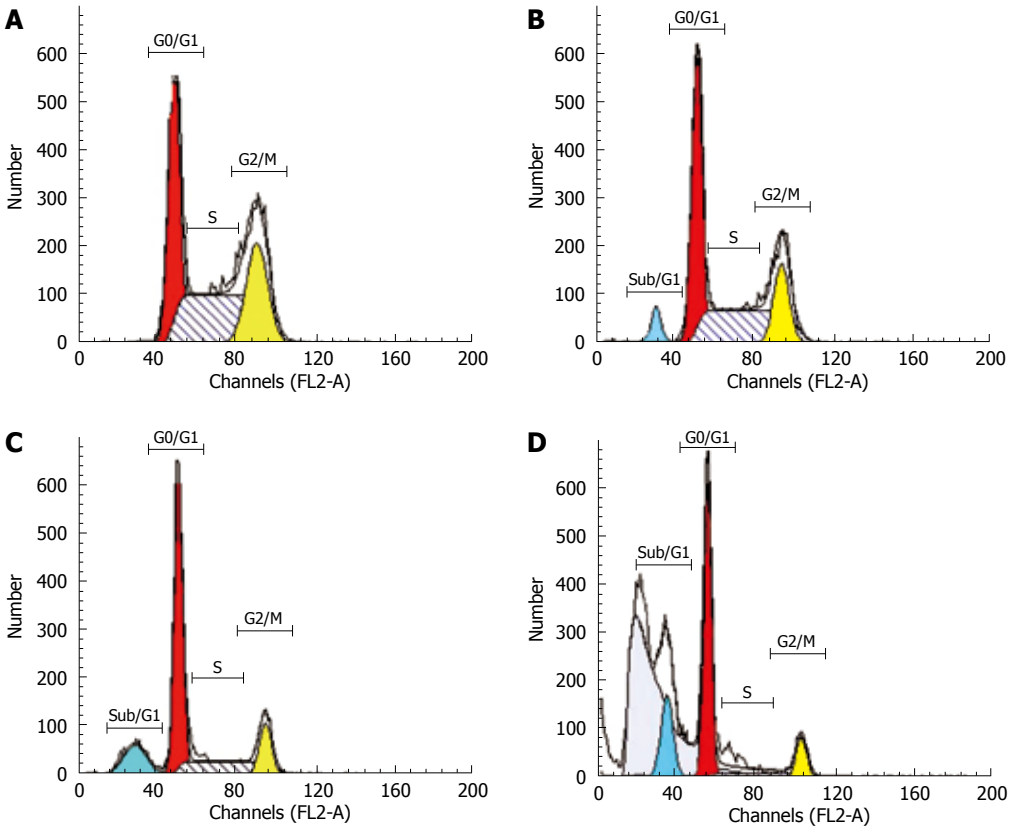

Figure 4 Effect of alisol B acetate on cell cycle distribution of SGC7901 cells. SGC7901 cells were treated with 0.1% DMSO (A) or with 30 μmol/L alisol B acetate for 24 h (B), 48 h (C), or 72 h (D). The cells were collected sequentially and stained with PI and analyzed by FCM.

regulation at 72 h after treatment that was associated with increased levels of Apaf-1, and activated caspases-3 and caspase-9. These results suggest that alisol B acetate induces SGC7901 apoptosis, at least partly, through up-regulation of pro-apoptotic Bax, resulting in up-regulation of the Bax/Bcl-2 ratio and activation of caspase-3 and caspase-9.

Effects of alisol B acetate on PI3K/Akt pathway

Akt, a major downstream target of PI3K, may be the best-characterized kinase known to promote cellular survival[13] and is dysregulated (mainly overexpressed) in a wide spectrum of human cancers, including gastric, hepatoma, and ovarian cancers[14-16]. To determine whether regulation of the Akt signal pathway is necessary for alisol B acetate-induced apoptosis, we investigated the expression of PI3K and Akt after treatment with 30 μmol/L Alisol B acetate. As shown in Figure 7, the levels of PI3K and phosphorylated Akt are time-dependently decreased in response to alisol B acetate. PI3K and phosphorylated Akt were rapidly decreased at 24 h, while the total Akt protein levels remained constant during alisol B acetate treatment. Therefore, these results suggested that Akt/PKB is associated with the survival of SGC7901 gastric cancer cells and inhibition of the PI3K/Akt signaling pathway increased the apoptosis induced by the alisol B acetate.

DISCUSSION

Apoptosis plays an important role in developmental processes, maintenance of homeostasis, and elimination of the damaged cells. Accumulated data indicate that many anticancer drugs can cause the death of tumor cells through the induction of apoptosis[15,17]. Alisol B acetate induces cell apoptosis in hepatoma and leukemia cells[7,8]. However, the effects of alisol B acetate on human gastric cells are still unclear. Therefore, the purpose of the present study was to find out the molecular mechanism of alisol B acetate underlying human gastric cancer cell line SGC7901.

In the present study, we first demonstrated that SGC7901 cells treated with alisol B acetate showed a dose- and time-dependent inhibition of the proliferation. Nuclear fragmentation, chromosome condensation, cell shrinkage and formation of apoptotic bodies were also observed. Flow cytometric analysis revealed that alisol B acetate treatment results in an increase of apoptotic cells. These results suggest that alisol B acetate-induced apoptosis contributes to the growth inhibition of SGC7901 cells.

Mitochondrion plays a critical role in apoptosis induced by some drugs[11]. The mitochondrial death pathway is associated with changes in the permeability of the outer mitochondrial membrane, the collapse of membrane potential[18,19]. Mitochondrial membrane permeability is mainly controlled by the Bcl-2 family of proteins, through regulation of the formation of apoptotic protein-conducting pores in the outer mitochondrial membrane[20-22]. Members of the Bcl-2 family proteins can be divided into two subfamilies; one is anti-apoptotic protein such as Bcl-2 and Bcl-X_L, the other is pro-apoptotic protein such as Bax, Bad, and Bid[23]. The expression of Bcl-2 and Bax is significantly involved in the balance of pro-apoptotic and anti-apoptotic signals at the mitochondrial level[24].

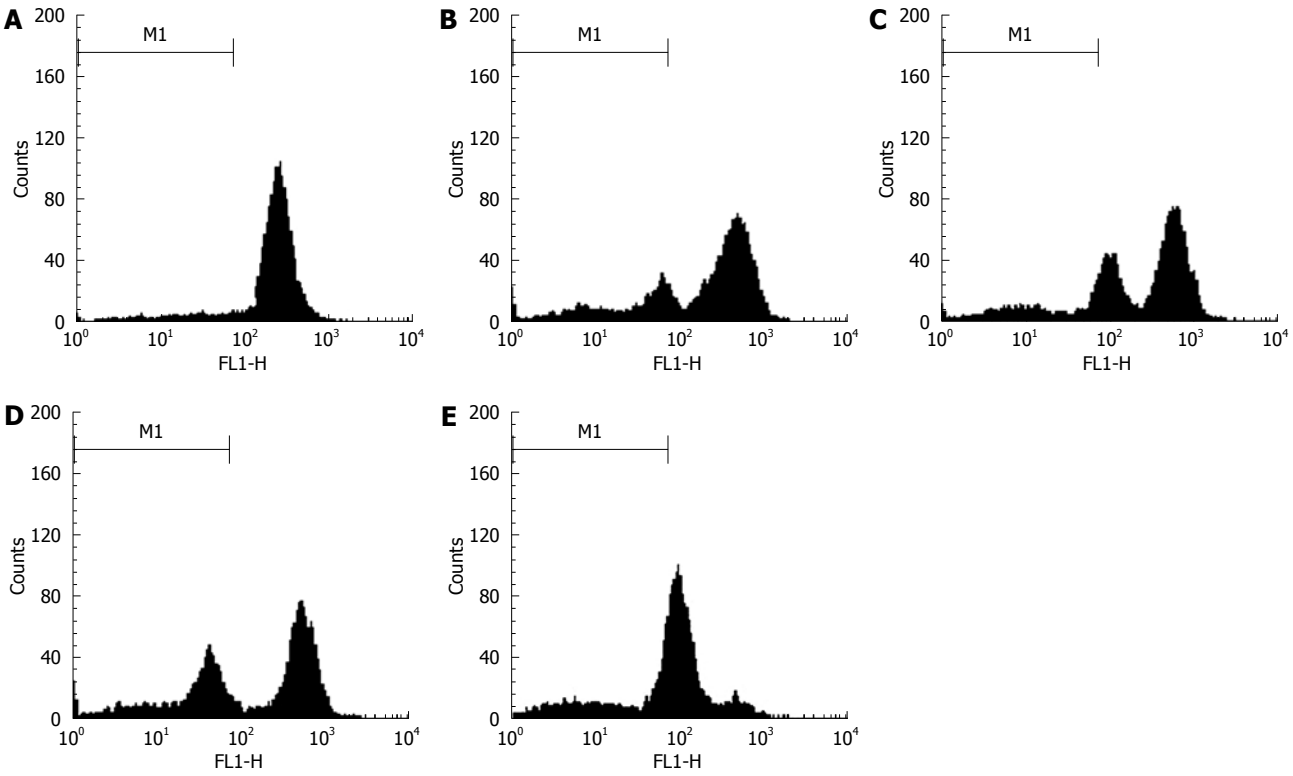

Figure 5 FCM analysis of mitochondrial membrane potential in human SGC7901 cells with 30 μmol/L alisol B acetate for various time periods. The zero concentration was defined as control. The percentage of cells stained with Rh-123 was determined by FCM as described in the Materials and Methods. A: Control; B-E: 12, 24, 48 and 72 h.

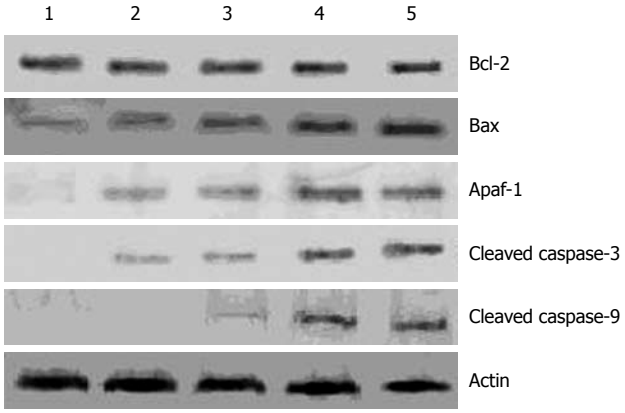

Figure 6 Effect of Alisol B acetate on the expression of Bcl-2, Bax, Apaf-1, cleaved caspase-3 and caspase-9. SGC7901 cells were treated with 30 μmol/L alisol B acetate for 12, 24, 48 and 72 h. The cells were collected and lysed. Western blotting analysis was conducted and probed with antibodies to Bcl-2, Bax, Apaf-1, caspase-3 and caspase-9. Lanes (from left to right): Control cells; 12 h; 24 h; 48 h; 72 h.

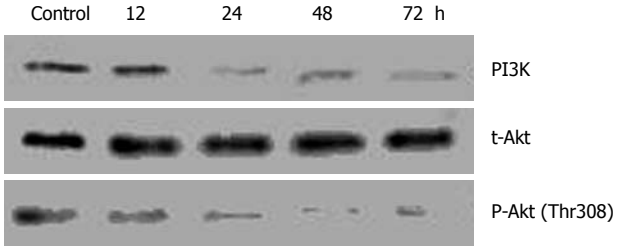

Figure 7 Effect of alisol B acetate on PI3K/Akt activation in SGC7901 cells. SGC7901 cells were treated with 0.1% DMSO (A) or 30 μmol/L alisol B acetate for 12, 24, 48 and 72 h. Western blotting analysis for PI3K, t-Akt and P-Akt was performed using specific antibodies with β-actin as a loading control.

The ratio of Bax/Bcl-2 is critical for the induction of apoptosis and this ratio determines whether cells will undergo apoptosis[25,26]. An increase in the ratio of Bax/Bcl-2 stimulates the release of cytochrome c from mitochondria into the cytosol. The cytosolic cytochrome c then binds to Apaf-1, leading to the activation of caspase-9, caspase-3 and poly (ADP-ribose) polymerase[27,28]. Thus, we examined the effect of alisol B acetate on Bax, Bcl-2 and mitochondrial membrane potential. In our experiments, alisol B acetate increased pro-apoptotic Bax expression without affecting Bcl-2 expression, leading to up-regulation of the ratio between pro-apoptotic (Bax) and anti-apoptotic (Bcl-2). This may be responsible for the concomitant execution phase of apoptosis that we observed, which included disruption of mitochondrial membrane potential.

Caspases, a family of cysteine proteases, are integral parts of the apoptotic pathway. Caspase-9 is the apical caspase in the intrinsic or mitochondria-initiated apoptosis pathway and requires the release of cytochrome C from the mitochondria as well as interactions with Apaf-1[18]. Activation of caspase-3 correlated with activation of caspase-9. Caspase-3 in particular, when activated, has many cellular targets[29]. During apoptosis, caspase-3 is one of the key executioners of apoptosis in response to various stimuli[30]. In many studies, it has been determined that a variety of chemotherapeutic agents induce apoptosis through the activation of caspases[15]. Previous studies have observed that alisol B

acetate induces apoptosis in human hepatoma Hep3B cells and human hormone-resistant prostate cancer PC-3 cells through an increase of caspase-3 and caspase-9 activity[7,9]. Consistent with an increase in the ratio of Bax/Bcl-2 and disruption of mitochondrial membrane potential, this study showed that alisol B acetate resulted in a time dependent up-regulation of Apaf-1, and activation of caspase-9 and caspase-3.

According to the present data, alisol B acetate may first increased the ratio of Bax/Bcl-2, which leads to disruption of mitochondrial membrane potential, and then activates mitochondria-mediated downstream molecular events including cytochrome c release and sequential activation of caspase-9 and caspase-3. These results demonstrated that a mitochondrial damage-mediated caspase pathway might be involved in alisol B acetate -induced apoptosis of SGC7901 cells.

The PI3K pathway regulates several cellular processes, including proliferation, growth[31], apoptosis[32] and cytoskeletal rearrangement. Akt, a major downstream target of PI3K, was originally reported as the cellular counterpart of the viral oncogene, which was amplified in gastric adenocarcinoma[14]. Overexpression of Akt isoforms has now been reported in ovarian, breast, prostate, and pancreatic cancers[16,33-35]. In addition, Akt activity is increased in cancers where the PTEN tumor suppressor is mutated[36]. In recent years, a number of cancer researchers have focused on the central importance of the PI3K/Akt pathway, and therapeutic strategies that target the PI3K/Akt pathway are now being developed[15,37]. PI3K consists of p85 regulatory subunit and a p110 catalytic subunit. Akt is activated by recruitment to the plasma membrane though direct contact of its Plecpstrinhomology domain with PIP3, and phosphorylation at Thr308 and Ser473. Thr308 is phosphorylated by the 3-phosphoinositide-dependent protein kinase PDKI[38]. Through its interaction with proteins in the Bcl-2 family such as BAD, Bax, Bcl-X_L and their downstream effectors, Akt provides a survival signal to the cell[39]. Additionally, Akt blocks cytochrome C release from the mitochondria through regulation of Bcl-2[40] and inhibits the catalytic activity of a pro-death protease, caspase 9 through phosphorylation[41].

In our study, we evaluated the effect of alisol B acetate on the PI3K/Akt pathways by measuring the PI3K and total Akt protein and sequential expression levels of phospho-Akt. We observed that alisol B acetate markedly inhibited PI3K and phospho-Akt after 24 h of treatment. No additional reduction in PI3K and phospho-Akt levels was seen when treatment was prolonged to 72 h and the total Akt protein levels remained constant throughout the course of the experiment. Meanwhile, our data showed that alisol B acetate treatment up-regulates Bax protein and down-regulates mitochondrial membrane potential. Furthermore, our data also showed activation of caspase-9 and caspase-3 in alisol B acetate-treated groups. Inhibition of PI3K/Akt signaling pathway is possibly preceded by modulations in Bax protein, leading to mitochondrial damage and activation of caspase-9, caspase-3 in favor of apoptosis.

In conclusion, we have demonstrated that alisol B acetate significantly induces apoptosis. This apoptotic response is associated with the up-regulation of the ratio of Bax/Bcl-2, loss of mitochondrial membrane and caspase activation. Moreover, the inhibition of PI3K/Akt pathway may play an important role in alisol B acetate-induced apoptosis. Therefore, we believe that alisol B acetate might be a promising molecule in cancer chemoprevention or chemotherapy; and further efforts to explore this therapeutic strategy are necessary.

COMMENTS

Background

Alisol B acetate is a major ingredient isolated from Alismatis rhizome. In recent years, alisol B acetate has shown various biological activities, including inhibitory effects on lipopolysaccharide, the inhibition of complementary activity, antibody-mediated allergic reaction and anti-tumor effects. However, its molecular mechanisms in the anti-tumor effects have not been well documented.

Research frontiers

Studies have confirmed that many Chinese herbs have anti-tumor properties and induce apoptosis. In the process of cell apoptosis induced by drugs, mitochondria plays a great role. phosphatidylinositol 3-kinases (PI3K)/Akt pathway is important in the development and proliferation of various human cancers. In recent years, a number of cancer researchers have focused on the importance of the PI3K/Akt pathway, and therapeutic strategies that target the PI3K/Akt pathway are being developed. In the present work, the authors investigated the effect of alisol B acetate on human SGC7901 cell proliferation, mitochondrial and PI3K/Akt signaling pathways.

Innovations and breakthroughs

The present study shows that alisol B acetate induces apoptosis and decreases proliferation in human gastric cancer cells. It was shown for the first time that alisol B acetate induced human gastric cancer apoptosis by modulation of the Bcl-2 family, loss of mitochondrial membrane potential, activation of caspases and inhibition of PI3K/Akt pathway.

Applications

The data of this article demonstrate the anti-cancer properties of alisol B acetate as well as its mechanism of action. By knowing the mechanism of action of alisol B acetate, it may provide a new therapeutic option, as a potential anticancer agent in the treatment of gastric cancer.

Terminology

PI3K is a major regulator of cell proliferation located in the Akt upstream, and together PI3K and Akt define a signal transduction pathway important in the pathogenesis of many different cancer types. Alisol B acetate is a natural herbal substance.

Peer review

This is a carefully performed study with novel findings that alisol B acetate has the potential for therapeutic application in gastric cancer. It examines the effect of alisol B acetate on the growth of human gastric cancer cell line and its possible mechanism of action. This paper is well-written. The experiments were well-designed and well-executed.

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BRIEF ARTICLES

Comparison of reflux esophagitis and its complications between African Americans and non-Hispanic whites

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Abstract

AIM: To determine the effect of ethnicity on the severity of reflux esophagitis (RE) and its complications.

METHODS: A retrospective search of the endoscopy database at the University of Florida Health Science Center/Jacksonville for all cases of reflux esophagitis and its complications from January 1 to March 31, 2001 was performed. Inclusion criteria were endoscopic evidence of esophagitis using the LA classification, reflux related complications and self-reported ethnicity. The data obtained included esophagitis grade, presence of a hiatal hernia, esophageal ulcer, stricture and Barrett's esophagus, and endoscopy indication.

RESULTS: The search identified 259 patients with RE or its complications, of which 171 were non-Hispanic whites and 88 were African Americans. The mean ages and male/female ratios were similar in the two groups. RE grade, esophageal ulcer, stricture and hiatal hernia frequency were likewise similar in the groups. Barrett's esophagus was present more often in non-Hispanic whites than in African Americans (15.8% *vs* 4.5%; $P < 0.01$). Heartburn was a more frequent indication for endoscopy in non-Hispanic whites with erosive esophagitis than in African Americans (28.1% *vs* 7.9%; $P < 0.001$).

CONCLUSION: Distribution of RE grade and frequency of reflux-related esophageal ulcer, stricture and hiatal hernia are similar in non-Hispanic whites and African Americans. Heartburn was more frequently and nausea/vomiting less frequently reported as the primary endoscopic indication in non-Hispanic whites compared with African Americans with erosive esophagitis or its complications. African Americans have a decreased prevalence of Barrett's esophagus compared with non-Hispanic whites.

Key words: Reflux esophagitis; African American; Hiatal hernia; Barrett's esophagus

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INTRODUCTION

Multiple studies suggest that the frequency of gastroesophageal reflux disease (GERD) complications such as erosive esophagitis, stricture and Barrett's esophagus (BE) is significantly lower in the US minority populations compared with non-Hispanic whites (nHw)[1-6]. Two studies from Veterans Affairs Medical Centers observed that severe GERD affected older nHw more commonly than non-whites[2,3]. Both of these investigations used the Department of Veterans Affairs patient treatment file as the data source.

Two groups of investigators have attempted to determine the frequency of GERD complications between ethnic groups seen at their institutions[7,8]. In one study[7], African Americans (AA), nHw and Asians significantly differed in heartburn prevalence, while the other[8] indicated that AA and nHw had equivalent heartburn prevalence rates. Both studies revealed that AA had decreased rates of erosive esophagitis compared with nHw. The prevalence of BE was only assessed in one investigation with the overwhelming majority seen in nHw.

Regarding BE, limited data exists about the prevalence of this entity in the United States minority populations. Initial studies compared non-Hispanic whites with African Americans. These revealed a predominant presence of BE in nHw when compared with AA[7-9]. In contrast, a report from the National Cancer Institute indicated that the incidence of esophageal adenocarcinoma is increasing in AA[10]. BE is considered the precursor lesion of esophageal adenocarcinoma, therefore one could speculate that the incidence of BE might also be increasing within the AA population.

There is minimal data evaluating the prevalence of GERD complications in any defined general population other than non-Hispanic whites[11,12]. The goal of this study is to compare the severity of reflux esophagitis and its complications in AA and nHw patients who underwent endoscopy at our institution.

MATERIALS AND METHODS

Patient population

A retrospective search of the endoscopy laboratory report database was performed to determine the total number of patients having upper gastrointestinal endoscopy (EGD) at the University of Florida Health Science Center/Jacksonville from 1 January to 31 March 2001. Inclusion criteria were endoscopic evidence of reflux esophagitis, esophageal stricture and/or ulcer, BE, and self reported ethnicity. Exclusion criteria were previous diagnostic EGD, history of non-GERD esophageal condition with the potential to cause esophageal injury (acquired immunodeficiency syndrome, esophageal infections, caustic ingestion, and thoracic radiation), and absence of demographic information within the patient record. The study was approved by the Institutional Review Board of the University of Florida Health Science Center/Jacksonville.

Symptom evaluation

Indication for EGD was recorded on the individual reports by all endoscopists. If multiple indications were listed, the first indication listed was used at the primary reason for performing the procedure. Patients with an indication such as follow-up of any previously noted esophageal lesion were not included in the analysis.

Classification of endoscopic findings

Esophagitis was graded using the Los Angeles system[13,14]. This scheme categorizes mucosal injury as follows: grade A defined as one or more mucosal breaks no longer than 5 mm which do not extend between the tops of two mucosal folds; grade B defined as one or more mucosal breaks more than 5 mm long that do not extend between the tops of two mucosal folds; grade C defined as one or more breaks that are continuous between the tops of two or more mucosal folds but involve < 75% of the esophageal circumference; and grade D defined as one or more mucosal breaks that involve at least 75% of the esophageal circumference. Four-quadrant biopsy specimens were taken at 2 cm intervals from any visible length of columnar lined esophagus to assess the presence of histologic BE. Biopsy sections were then stained with Alcian blue to detect the presence of specialized intestinal metaplasia, the characteristic feature of BE.

Esophageal stricture was defined as a narrowing of the esophageal lumen, which either did not allow passage of a 9 mm endoscope or allowed distal passage of the endoscope with difficulty. The length of the narrowing was measured using the 5 cm endoscope length markings as a reference. Hiatal hernia was defined as the presence of gastric mucosa above the level of the esophageal hiatus. The hernia length was measured using the 5 cm markings as a reference. Hiatal hernia length was considered small if < 2 cm, medium from 2 to 5 cm, and large if greater than 5 cm. The hernia was defined as sliding if the stomach re-entered the abdominal cavity during the endoscopy. Esophageal ulcer was defined as an excavation of the esophageal mucosa of at least 3 mm wide. The ulcer margin had to be raised from the base by at least 1 mm. Location of the ulcer was noted as distal, mid or proximal esophagus.

Table 1 Demographic data of the study population

	AA (n = 88)	nHw (n = 171)
Male/female (%)	38/62	48/52
Age range (yr)	26-101	18-90
Mean age (mean ± SD)	58.5 ± 16.9	55.8 ± 14.5

P = NS for all, NS represents not significant.

Statistical analysis

All values of esophagitis distribution frequency, presence of stricture, esophageal ulcer, hiatal hernia, BE and procedure indication were reported as percentage present for each group. Student's t-test was used for comparisons between groups. Differences between groups will be considered significant if $P < 0.05$. Data analysis was performed using JMP 5.0 for Windows (SAS Institute Inc., Cary, NC).

RESULTS

Demographics of the study population

During the study period, 716 patients had an EGD and 259 of the 716 patients met the criteria for study inclusion. Of the study group, 171 (66%) were nHw and 88 (34%) were AA. Males comprised 48% of the nHw and 38% of the AA groups, respectively. The groups were similar in age and gender distribution (Table 1).

Indication for endoscopy

Table 2 illustrates the indication for EGD between ethnic groups. Heartburn was noted as the primary indication significantly more frequently in nHw than in AA patients (nHw 28.1% and AA 8%; $P < 001$). Nausea and/or vomiting were noted significantly more frequently in AA than nHw patients (nHw 2.9% and AA 9%; $P < 0.04$). Other indications for the procedure were similar between ethnic groups.

Table 2 Indication for endoscopy by ethnicity *n* (%)

Indication	88 AA patients	171 nHw patients	*P* value
Heartburn	7 (8)	48 (28.1)	< 0.001
Dysphagia	14 (15.9)	37 (21.6)	NS
Upper GI bleeding	22 (25)	25 (14.6)	NS
Nausea/vomiting	8 (9.1)	5 (2.9)	< 0.040
Abdominal pain	15 (17)	31 (18.1)	NS
Abnormal X-ray	3 (3.4)	4 (2.3)	NS
Anemia	8 (9.1)	10 (5.9)	NS
Weight loss	4 (4.5)	2 (1.2)	NS
Other	7 (8)	9 (5.3)	NS

Reflux esophagitis grade distribution

Of the 259 patients, 204 had evidence of reflux esophagitis on EGD. Of those with esophagitis, 76 were AA and 128 were nHw. The distribution of esophagitis grade among those with esophagitis in both ethnic groups is illustrated in Table 3. There was no difference observed between the two groups regarding the severity of erosive esophagitis seen.

Table 3 Distribution of reflux esophagitis grade by ethnicity

Ethnicity	LA classification grade			
	A	B	C	D
AA (%) (*n* = 76)	63.2	18.4	7.9	10.5
nHw (%) (*n* = 128)	71.1	12.5	7.0	9.4

P = NS.

Table 4 Prevalence of other endoscopic findings by ethnicity *n* (%)

	AA patients	nHw patients	*P* value
Hiatal hernia	18 (20.4)	39 (22.8)	NS
Stricture	2 (2.2)	10 (5.8)	NS
Ulcer	7 (7.9)	12 (7)	NS
Endoscopic Barrett's esophagus	4 (4.5)	27 (15.8)	< 0.01
Histological Barrett's esophagus	0 (0)	9 (5.3)	0.03

Prevalence of other esophageal findings

Table 4 demonstrates the prevalence of hiatal hernia and endoscopic complications of GERD observed between ethnic groups. With regard to presence of hiatal hernia, esophageal stricture and ulcer, no difference was observed between the groups. However, endoscopic and histologic BE were present significantly more frequently in nHw than AA patients (endoscopic BE: nHw 15.8% and AA 4.5%, $P < 0.01$; histologic BE: nHw 5.8% and AA 0%, $P < 0.04$).

DISCUSSION

The current study comparing the spectrum of reflux esophagitis between nHw and AA patients was designed to test the hypothesis that reflux esophagitis severity and its complications vary between nHw and AA at EGD. The results indicate that the distribution of severity and reflux related complications, other than BE, are similar between AA and nHw patients. This similarity in reflux esophagitis severity, presence of hiatal hernia and complications of reflux esophagitis (except for BE) observed between ethnic groups has not been previously described[2,7,8]. Also, the primary indication for the diagnostic procedure (heartburn in nHw and upper GI bleeding in AA) revealed differences between AA and nHw patients not noted previously. Both of these factors provide insight into reflux disease within the Unites States as a whole and specifically in the African American community.

Similarities and differences in reported GERD symptoms between ethnicities have been reported previously[7,8]. Spechler and colleagues noted that AA complained, understood and met predefined criteria for heartburn more frequently than was observed in either nHw or Asian patients in the metropolitan Boston area. However, El Serag and associates observed that the occurrence of weekly heartburn and/or regurgitation was no different between AA, nHw and a multiethnic group who were all employees at the Houston VA Medical Center. Complicating this further was the use of a survey (GERQ) in the investigation of El Serag *et al* to assess GERD symptoms, which had only been previously validated in nHw or Spaniards[15,16]. It is well recognized that medical communication differs between AA and nHw[17]. Using a tool not validated in African Americans might have led to underestimation of the prevalence of reflux symptoms in that group. This is also suggested by the difference in clinical indication for EGD observed between AA and nHw in the present investigation.

The difference observed in the prevalence of endoscopic BE between AA and nHw in the present investigation corresponds with previous reports in the literature[7,18,19]. The prevalence of histologically confirmed BE among nHw patients is also consistent with the single publication that specifically addressed that issue[19]. The finding that endoscopic BE was only confirmed in 1/3 of cases strongly supports the need for histology in assessing BE.

There are limitations of this investigation that should be recognized. Only those who were referred and presented for EGD were eligible for inclusion in this study. As suggested by previous reports[7,8], AA patients may have not been referred for endoscopy as frequently. This could have led to an underestimation of reflux-related endoscopic disease in that group. The effect of obesity was not accounted for in our study. Multiple studies have indicated an association linking increasing body mass index and presence of reflux symptoms in women, in addition to existence of obesity as a likely risk factor in males[20,21]. A negative association between *H pylori* presence and GERD symptoms is well established[22]. Unfortunately, biopsies of the antrum were not routinely taken in our study, therefore the presence and impact of *H pylori* colonization could not be adequately assessed.

In summary, the results of this study indicate that AA have a similar distribution of esophagitis severity and complications from GERD when compared with nHw. However, the presence of Barrett's esophagus is more common in nHw than AA. Also, it appears that AA and nHw patients with reflux esophagitis and its complications on endoscopy have different indica-

tions for EGD. The reasons for the observed difference in procedure indication and the development of histologic Barrett's esophagus between racial groups are not currently known. However, our data suggests that ethnicity may influence both symptoms leading to endoscopy and the development of BE, a premalignant change in the esophagus due to GERD. Further ethnic-specific investigations are needed to completely understand the lower prevalence of Barrett's esophagus between African Americans and non-Hispanic whites.

COMMENTS

Background

There is minimal data evaluating the prevalence of gastroesophageal reflux disease (GERD) complications in any United States general population other than non-Hispanic whites (nHw). Presently, it is thought that such complications occur less frequently in African Americans (AA) than non-Hispanic whites.

Research frontiers

Barrett's esophagus is a well-recognized complication of GERD and is the principal risk factor for esophageal adenocarcinoma. It is well known that Barrett's esophagus is more frequently discovered in nHw than in other United States ethnic groups. However, differences in other GERD associated conditions between ethnic groups have not been well evaluated. In this study, the authors demonstrate reflux esophagitis and its complications, except for Barrett's esophagus, occur in an equal distribution between AA and nHw patients.

Innovations and breakthroughs

Recent reports have highlighted discrepancies in the prevalence of Barrett's esophagus. However, minimal data exists on the frequency of reflux esophagitis and its complications in communities with a significant component of African Americans. This is the first study to report that reflux esophagitis and its complications, other than Barrett's esophagus, occur at a similar frequency in nHw and AA. In addition, indication for the index endoscopy appears to be different in the above ethnic groups.

Applications

By understanding GERD and its complications among ethnic groups in the United States, this study might indicate future avenues for investigation to prevent the development of Barrett's esophagus and esophageal adenocarcinoma.

Terminology

Barrett's esophagus is the antecedent neoplastic lesion associated with the development of esophageal adenocarcinoma. Incidence of esophageal adenocarcinoma is 3-4 times more frequent in non-Hispanic whites than African Americans.

Peer review

In this paper the authors evaluated the effect of ethnicity on the severity of reflux esophagitis. It deals with an important subject. However, as an endoscopic study, it is limited by lack of information regarding body mass index, access to medication, type of medication used for GERD and socioeconomic status of the different ethnic groups seen.

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BRIEF ARTICLES

Factors associated with patient absenteeism for scheduled endoscopy

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Abstract

AIM: To identify risk factors to help predict which patients are likely to fail to appear for an endoscopic procedure.

METHODS: This was a retrospective, chart review, cohort study in a Canadian, tertiary care, academic, hospital-based endoscopy clinic. Patients included were: those undergoing esophagogastroduodenoscopy, colonoscopy or flexible sigmoidoscopy and patients who failed to appear were compared to a control group. The main outcome measure was a multivariate analysis of factors associated with truancy from scheduled endoscopic procedures. Factors analyzed included gender, age, waiting time, type of procedure, referring physician, distance to hospital, first or subsequent endoscopic procedure or encounter with gastroenterologist, and urgency of the procedure.

RESULTS: Two hundred and thirty-four patients did not show up for their scheduled appointment. Compared to a control group, factors statistically significantly associated with truancy in the multivariate analysis were: non-urgent *vs* urgent procedure (OR 1.62, 95% CI 1.06, 2.450), referred by a specialist *vs* a family doctor (OR 2.76, 95% CI 1.31, 5.52) and office-based consult prior to endoscopy *vs* consult and endoscopic procedure during the same appointment (OR 2.24, 95% CI 1.33, 3.78).

CONCLUSION: Identifying patients who are not scheduled for same-day consult and endoscopy, those referred by a specialist, and those with non-urgent referrals may help reduce patient truancy.

Key words: Absenteeism; Colonoscopy; Endoscopy; Esophagogastroduodenoscopy; Gastroenterologist

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INTRODUCTION

Patient absenteeism from scheduled outpatient appointments is a major problem for all ambulatory clinics. Failure to attend an appointment results in inefficiency because the vacant appointment interval is often not used by another patient. This typically results in ongoing expenditures without concomitant reimbursement thereby decreasing appropriate resource utilization. This is particularly important for endoscopic procedures where a specific interval is scheduled and appropriate preparation is required for each patient. If a patient does not attend the prearranged endoscopy time, often the time is simply absorbed into the rest of the day without an appropriate substitute being found.

There are a number of maneuvers that various clinics have used in an attempt to decrease truancy from endoscopic appointments. Some sites notify all patients within a few days of their scheduled time, others will insist that the patient themselves confirm their appointment, other sites even "over-book" the endoscopy unit to account for a small percentage that will not appear on their scheduled day. Various methods such as telephone[1] or text message[2] reminders and mailed pre-procedural pamphlets[3] have been used successfully to decrease truancy from endoscopic appointments. Very little research has been devoted to enhancing our

understanding of why patients do not appear at their scheduled appointments, and that which has been done has demonstrated conflicting results[4-8].

Research based in adolescent outpatient clinics have found that telephone reminders the day before the scheduled appointment help to reduce "no-show" rates[9]. Other studies have shown that such reminder systems do not improve patient attendance rates[10,11]. One prospective study found that previous non-attendance for an outpatient appointment was the strongest predictor of future non-attendance behavior[8]. There has been limited research into the explanations or reasons for patient absenteeism for scheduled gastroenterology appointments[12]. If patients could be identified as "high-risk" for absenteeism, then specific targeted efforts could be developed to ensure their appropriate appearance at their procedure. The objective of this study is to identify risk factors that may help predict which patients are the most likely to be truant for a scheduled elective endoscopic procedure.

MATERIALS AND METHODS

This study involved all consecutive patients scheduled to undergo an elective esophagoduodenoscopy (EGD), flexible sigmoidoscopy or colonoscopy in a single Canadian tertiary care, gastroenterology clinic (hospital based) in the year 2003. A retrospective chart review was performed to identify all patients who did not appear for their scheduled outpatient endoscopic procedure and they were compared to a control group (randomly selected patients from the same time period who did show at their appointment) to generate predictors of patient absenteeism. It was felt that it was unnecessary, and in fact not practical, to assess all patients during the entire year that did appear for their examination. By using a random sample (selected from a similar time period as the truant group) comparison between the two groups was deemed statistically appropriate. Patients referred from other hospitals and those undergoing endoscopic retrograde cholangiopancreatography (ERCP) or endoscopic ultrasound (EUS) were excluded.

The factors analyzed included gender, age, duration of time on a waiting list, time of day of procedure (07:30-10:00, 10:00-12:30, 12:30-15:00), day of the week, type of procedure, referring physician (family physician *vs* other specialist), distance to hospital (divided into regional areas), whether the patient went direct to the endoscopy suite for a consult and endoscopy during the same appointment without consulting the gastroenterologist in his/her outpatient clinic prior to the procedure, urgency of the procedure (urgent procedures were defined as patients who were bleeding or who had radiological abnormalities warranting an endoscopic procedure) and whether the patient was undergoing a repeat procedure by the previous gastroenterologist or surgeon. Univariate analysis was then performed to determine independent associations of each factor to patient "no-shows". Odds ratios (OR), 95% confidence interval (95% CI) and their respective *P*-values were generated. Following this, multivariate analysis was performed using logistic regression analysis. Only factors that were statistically significant in multivariate analysis were reported in the model. The SPSS software package for Windows (Release 15.0.0-6 Sept 2006; SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Ethics approval was obtained through St Paul's Hospital, University of British Columbia to conduct the study.

Table 1 Prevalence of patient characteristics among patient's who were and were not truant for scheduled endoscopic procedure

Factor		Number of patients (*n*)		
		Control group[1]	No-show	Total patients evaluated
Urgent procedure	No	219	198	417
	Yes	99	50	149
Direct to endoscopy	No	235	187	422
	Yes	81	75	156
Referring doctor	No referral	9	17	26
	GP	293	235	528
	Specialist	19	33	52
Sex	Female	176	152	328
	Male	150	133	283
Time of procedure	7:30-10:00	125	87	212
	10:00-12:30	115	106	221
	12:30-15:00	86	92	178
Type of procedure	Colonoscopy	177	129	306
	EGD	109	107	216
	Flex-sig	40	49	89
Weekday	Monday	53	61	114
	Tuesday	58	65	123
	Wednesday	72	56	128
	Thursday	73	61	134
	Friday	70	42	112
Distance living from hospital	Within 10 miles	201	194	395
	Within 60 miles	106	74	180
	Beyond 60 miles	18	13	31
Previous endoscopy	No	132	104	236
	Yes	184	144	328
New patient	No	153	135	288
	Yes	161	133	294

[1]Control group refers to a random sample of patients during the same interval who appeared at their appointment as scheduled; GP: General practitioner; Flex-sig: Flexible sigmoidoscopy.

RESULTS

Table 1 shows the prevalence of patient characteristics that were analyzed as potential predictive factors for truancy for scheduled endoscopic procedure. The absenteeism rate was 3.6% (n = 234) overall, 2.6% for colonoscopy, 2.9% for EGD and 4.3% for flexible sigmoidoscopy. Of the 234 patients, 50% were scheduled for colonoscopy, 35% for EGD and 15% for flexible sigmoidoscopy.

Univariate analysis was performed on each factor to determine the possibility of independent associations with patient "no shows" (Table 2, Figure 1) In the

Table 2 Univariate analysis of patient characteristics and their predictive value for patient absenteeism from scheduled endoscopic procedure

	Comparison	OR	95% CI	*P*-value
Urgency	Non-urgent *vs* urgent	1.790	1.211, 2.645	0.0035
Direct to endoscopy	Not direct *vs* direct	0.859	0.595, 1.242	0.4199
Referring physician	Specialist *vs* GP	2.165	1.200, 3.906	0.0063
	No referral *vs* GP	2.355	1.031, 5.379	
Age	Increments of 10 years	0.912	0.823, 1.010	0.0757
Sex	Female *vs* male	0.974	0.708, 1.340	0.8714
Time of procedure	7:30-10:00 *vs* 12:30-15:00	0.651	0.423, 1.001	0.0835
	10-12:30 *vs* 12:30-15:00	0.967	0.637, 1.468	
Type of procedure	Colonoscopy *vs* flex-sig	0.741	0.439, 1.250	0.2757
	EGD *vs* flex-sig	0.964	0.558, 1.667	
Weekday	Tues *vs* Mon	1.000	0.580, 1.723	0.9570
	Wed *vs* Mon	0.754	0.439, 1.293	
	Thurs *vs* Mon	0.809	0.476, 1.373	
	Fri *vs* Mon	0.493	0.278, 0.873	
Region	< 10 mile radius *vs* < 60 mile radius	1.537	0.685, 3.448	0.1110
	< 60 mile radius *vs* > 60 mile radius	1.054	0.454, 2.449	
Previous endoscopy	Yes *vs* no	1.039	0.738, 1.464	0.8252
Waiting time	3 mo increments	0.835	0.675, 1.034	0.0980
New patient	No *vs* yes	1.293	0.921, 1.815	0.1369

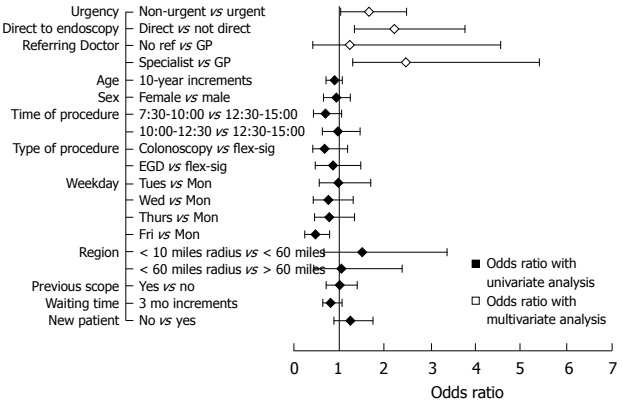

Figure 1 Predictive value for patient absenteeism of variables assessed in univariate (black lines) and multivariate (white lines) analyses. Three factors were statistically associated with patient absenteeism in the multivariate analysis: (1) patients referred for non-urgent *vs* urgent procedures (OR 1.62, 95% CI 1.06, 2.50); (2) patients referred by a specialist *vs* a family doctor (OR 2.76, 95% CI 1.38, 5.52); and (3) patients undergoing office-based consult prior to endoscopy *vs* consult and endoscopic procedure during the same appointment (OR 2.24, 95% CI 1.33, 3.78). In the univariate analysis, patients were more likely to show up for their scheduled appointment on Fridays, however, this was not significant in the multivariate analysis. GP: General practitioner; Flex-sig: Flexible sigmoidoscopy.

univariate analysis, a significant trend was determined towards truancy in those non-urgently referred (OR 1.79, 95% CI 1.2-2.6) and those referred from specialists (as opposed to family physicians) (OR 2.1, 95% CI 1.2-3.9). Interestingly, in the univariate analysis, having their procedure performed on Friday (as opposed to Monday to Thursday) was protective against truancy (OR 0.493, 95% CI 0.28-0.87).

Multivariate analysis was then performed to determine which factors were most associated with a positive outcome (Table 3, Figure 1). In the multivariate analysis three factors were statistically significant determinants in predicting "no shows": (1) patients referred to the clinic for a non-urgent compared to urgent procedures (OR 1.624, 95% CI 1.06, 2.45); (2) patients referred by a specialist compared to those referred by a family doctor (OR 2.76, 95% CI 1.31, 5.524); (3) patients who had an office-based consult prior to the endoscopy as compared to those who went direct to the endoscopy suite for a consult and procedure during the same appointment (OR 2.244, 95% CI 1.33, 3.78). In the multivariate analysis, day of the week the procedure was performed was no longer significant. Figure 1 summarizes the findings of the univariate analysis and the significant factors on the multivariate analysis.

Table 3 Multivariate analysis of patient characteristics and their predictive value for patient absenteeism from scheduled endoscopic procedure

	Comparison	OR	95% CI	*P*-value
Urgency	Non-urgent *vs* urgent	1.624	1.056, 2.497	0.027
Direct to endoscopy	Not direct *vs* direct	2.244	1.331, 3.783	0.002
Referring physician	Specialist *vs* GP	2.763	1.383, 5.519	0.058
	No referral *vs* GP	1.228	0.321, 4.706	0.666

DISCUSSION

There are many factors that are critical in maintaining the efficiency of an endoscopy unit. Many of these factors, such as emergency procedures, equipment failures and sedation difficulties are virtually impossible to predict. The multitasking, late physician is another major cause of an inefficient endoscopy unit and likewise, he/she is admittedly difficult to modify. On the other hand, truancy among patients who fail to attend their scheduled appointment is something that, in theory at least, has the capacity to be controlled.

We have determined the absenteeism rate in our endoscopy unit to be 3.6%, which encompassed 4.3% of flexible sigmoidoscopies, 2.6% of colonoscopies and 2.9% of upper endoscopies. This study was not

designed to compare the absenteeism rates between the different procedures; however, when compared, these rates are not statistically different. We determined three factors that resulted in a patient being considered "high risk" for truancy. It has become common for patients to have a consultation and endoscopy at the same time, just prior to the endoscopy (particularly for screening colonoscopies), and we have found that these patients are more likely to attend their appointment than those who have been previously seen in an office setting by the physician. At present, in our setting, we do not use physician assistants, however, all patients scheduled for a consult and endoscopic procedure at the same time are called by a secretary with subsequent explanation of the preparation and procedure. Those patients who continue to have additional questions that cannot be answered by the secretary are scheduled for an office visit prior to the procedure. The practice of "direct to endoscopy" has become commonplace and we note that this practice has indeed apparently minimized our truancy rates. It should also be recognized that in our Canadian system, many patients are booked for their endoscopic procedures weeks to months in advance and yet, despite this, we still have improved attendance rates for those patients that are booked directly to endoscopy, possibly demonstrating a more motivated patient group. It does confirm that this practice at least results in appropriate attendance to endoscopy and that these patients are compliant with their appointment.

The second factor found to be associated with absenteeism from endoscopy was referral from a specialist rather than a family physician. This is logical in that family physicians are very accustomed to referring patients to a specialist and have an organized system to arrange it. On the other hand, many specialists' offices are very adept at accepting referrals but not nearly as organized when it comes to referring a patient to another specialist. In addition, patients referred from other specialists often tend to have multiple health issues and more likely to be at a more acute state of illness. Just the fact that they have multiple health problems may put them at risk for absenteeism from their scheduled endoscopic appointment. This is another group of patients that can relatively easily be targeted as "high risk" for absenteeism and steps taken to ensure confirmation of their appointment.

The last group of patients who are more likely not to attend their appointments are those with non-urgent reasons for endoscopy. We defined urgent as those patients with bleeding or radiological abnormalities requiring endoscopic assessment. These patients are more likely to attend their procedure as opposed to the truly elective patient. This is logical in that typically, these patients have been told that there is a high likelihood that an abnormality is present and tissue confirmation is critical. These patients are therefore concerned enough to ensure their attendance at their endoscopic examination.

An Australian study demonstrated that patients with previous history of non-attendance were more likely not to attend[8], we have not found that in this study. This may be because if a patient doesn't attend the endoscopy clinic at a scheduled time, typically, the physician will not arrange another endoscopy until another office visit has been completed and an explanation for truancy extracted. A pediatric study demonstrated that social factors (social class, unmarried parents, poorer housing) played a larger role in increasing truancy than other factors such as severity of disease[13]. Due to the nature of this study, assessment of social factors was not performed.

There are several limitations of our study. It is retrospective and contains the usual limitations inherent within this study design. On the other hand, there is presently very limited data available from the literature to determine who is at high risk for truancy from endoscopy units. Many endoscopic sites have instituted measures to limit truancy such as calling all patients by phone or mailing reminders prior to the endoscopic examination to ensure their attendance[1-3,7,14]. Some of these measures are labor intensive with associated cost expenditures. Additionally, most patients attend their clinic appointment and in theory, don't require a reminder. If a select group of patients could be targeted then a limited reminder protocol might be considered. Before we embarked upon any campaign to decrease truancy rates, we felt it was critical to determine what factors were important in this area. Ideally, if we could isolate several factors, steps could be undertaken to improve the system and then re-evaluate after institution of an improved management strategy.

Another limitation of our study is the fact that it applies only to the dataset of our institution and our patients. Its general, applicability may be questioned; however, our site is very similar to many tertiary care centers. Many patients come directly to the endoscopy unit without prior consultation, procedures are performed in large numbers with rapid turnover, the endoscopic rooms and time are the critical elements to the efficiency of any unit. As a tertiary care center with a wide base of referrals, it would appear that our unit is, in fact, similar to many other endoscopic units throughout the world and therefore, our results could likely be replicated elsewhere.

A final limitation of the study is the fact that we have excluded patients who were transferred from other hospitals as well as those scheduled for ERCP and EUS. These patients are more complex with a myriad of other issues (including the acuity of illness) and we felt that the group we needed to concentrate on was those in whom we perform most of the standard, elective endoscopic examinations.

In summary, we found that patients with a non-urgent condition, those referred from a specialist and those who do not have a consult and procedure at the same time are more likely to be absent from their scheduled endoscopic procedure than those without these characteristics. With this information, endoscopy units can hopefully modify their clinical practices to reduce patient truancy. Studies aimed at improving

efficiency in endoscopy units should be aware of these "high-risk" patients to enhance appropriate resource utilization by decreasing absenteeism.

COMMENTS

Background

Patient absenteeism of outpatient procedures is a major problem for all ambulatory clinics.

Research frontiers

Identifying patients who may not present for scheduled endoscopy may help gastroenterologists to reduce patient truancy.

Innovations and breakthroughs

Previous studies have shown that patients at highest risk for truancy are those with a history of truancy; and methods to enhance patient attendance at clinics (i.e. telephone reminders, mailed reminders) have had mixed results.

Applications

Identifying patients who are not scheduled for same-day consult and endoscopy, those referred by a specialist rather than a family physician, and those with non-urgent reasons for referral may help gastroenterologists to reduce patient truancy.

Peer review

This manuscript is of enough interest and describes original work that merits its publication in WJG.

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Lower *Bifidobacteria* counts in both duodenal mucosa-associated and fecal microbiota in irritable bowel syndrome patients

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Abstract

AIM: To determine the composition of both fecal and duodenal mucosa-associated microbiota in irritable bowel syndrome (IBS) patients and healthy subjects using molecular-based techniques.

METHODS: Fecal and duodenal mucosa brush samples were obtained from 41 IBS patients and 26 healthy subjects. Fecal samples were analyzed for the composition of the total microbiota using fluorescent *in situ* hybridization (FISH) and both fecal and duodenal brush samples were analyzed for the composition of bifidobacteria using real-time polymerase chain reaction.

RESULTS: The FISH analysis of fecal samples revealed a 2-fold decrease in the level of bifidobacteria (4.2 ± 1.3 *vs* 8.3 ± 1.9, $P < 0.01$) in IBS patients compared to healthy subjects, whereas no major differences in other bacterial groups were observed. At the species level, *Bifidobacterium catenulatum* levels were significantly lower (6 ± 0.6 *vs* 19 ± 2.5, $P < 0.001$) in the IBS patients in both fecal and duodenal brush samples than in healthy subjects.

CONCLUSION: Decreased bifidobacteria levels in both fecal and duodenal brush samples of IBS patients compared to healthy subjects indicate a role for microbiotic composition in IBS pathophysiology.

Key words: Irritable bowel syndrome; Gut microbiota; Bifidobacteria; *Bifidobacterium catenulatum*

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INTRODUCTION

Irritable bowel syndrome (IBS) is a common gastrointestinal disorder characterized by abdominal pain or discomfort and altered bowel function. Alterations in psychosomatic factors, gastrointestinal motility, visceral hypersensitivity and microbiotic composition have been suggested to play a role in the pathophysiology of IBS[1]. Alterations in fecal and small intestinal microbiotic composition in IBS patients have been reported and studies revealed a somewhat higher bacterial count in jejunal juice of IBS patients and lower numbers of fecal coliforms, lactobacilli and bifidobacteria than in healthy subjects[2,3]. More specifically, molecular-based methods showed that in IBS patients levels of members of the *Clostridium coccoides* subgroup, *Lactobacillus*, *Collinsella* and *Bifidobacterium catenulatum* groups are different from that of healthy subjects[4,5]. These differences in the fecal microbiotic composition may underlie

symptom generation by promoting abnormal colonic fermentation[6]. However, mucosa-associated bacteria might be more relevant in the symptom generation of IBS, since fecal and jejunal juice samples are only representing the composition of luminal microbiota. Alterations in luminal bacteria composition may change the commensal microbiota and affect the microbiota adhering to the mucosa. Microorganisms adhering to the intestinal wall are more likely to affect the host's immune, physiological or neuronal system or vice versa. The composition of luminal and mucosa-associated bacteria are not the same since the micro-environments are different at the surface of the intestinal epithelium and the lumen[7,8]. Therefore, we aimed to determine the composition of fecal luminal and mucosa-associated microbiota in IBS patients using molecular identification and quantification techniques.

MATERIALS AND METHODS

Subjects

Twelve male and 29 female IBS patients included in this study fulfilled the Rome Ⅱ criteria for IBS and were categorized as diarrhea predominant (IBS-D), constipation predominant (IBS-C) or alternating IBS subgroup (IBS-A)[9]. The IBS population consisted of 14 IBS-D subjects, 11 IBS-C subjects and 16 IBS-A subjects. The control group consisted of 8 male and 18 female healthy subjects from the general population, devoid of GI symptoms or major abdominal surgery. The healthy subjects were significantly ($P < 0.001$) younger (31 ± 2.06 years) than the group of IBS patients (42 ± 2.12 years). Subjects taking medication known to influence bacterial composition and gastrointestinal motility, especially antimicrobial medications and/or probiotics were excluded from the study. The Human Ethics Committee of the University Medical Centre Utrecht approved the study and all subjects gave written informed consent.

Sampling

To obtain small intestinal mucosa-associated material a sterile cytology brush (Uno-Brush, Prince Médical, Ercuis, France) sheathed in a sterile catheter was placed through the endoscope biopsy channel and advanced under direct vision out beyond the endoscope tip[10]. The duodenal mucosa was brushed three times and then pulled back into the sheath of the catheter. The catheter was removed and the brush was immediately cut off the catheter and placed into a sterile tube in liquid nitrogen and stored at -80℃ until analysis. Fecal samples, obtained before endoscopy, were collected and stored at -80℃ until further handling.

Fluorescent in situ hybridization (FISH) analysis of fecal samples

The total number of bacteria present in the fecal samples was determined with the EUB 338 probe which targets all bacteria[11]. FISH analysis was essentially performed as described previously with the genus-specific probes listed in Table 1[12]. Approximately 0.5 g of homogenized feces was suspended in 4 mL of 0.2 mm-pore-size-filtered PBS and 0.5 mL 37% formaldehyde and thoroughly mixed by vortexing for 3 min. After incubation for 4 h at 4℃ the suspension was vortexed again for 2 min. Debris was removed by a short spin at 80 *g* for 1 min. In an eppendorf tube, 300 μL of the supernatant was collected and the fixed cells were washed twice with PBS. For FISH analysis of the *Lactobacillus-Enterococcus* group the cells were first permeabilized by resuspending the pellet in 100 μL Proteinase-K solution (180 kU/L) (Sigma-Aldrich, Zwijndrecht, The Netherlands) and incubated for 10 min at 37℃. The cells were washed as described above and resuspended in 300 μL PBS/ethanol (1:1 v/v). After one hour of storage at -20℃, the cell suspension was diluted 1:10 in hybridization buffer at the required temperature for hybridization, and 5 ng labeled probe was added. Cells were hybridized for 16 h at the prescribed hybridization temperature. After resuspension in 4 mL washing buffer, cells were filtered on a 0.2 mm pore size Isopore polycarbonate membrane filter (Millipore Corporation) and washed with 5 mL of 50℃ washing buffer. Filters were mounted on microscope slides with Vectashield (Vector Laboratories, Burlingame, CA, USA), and hybridized cells were counted visually using an Olympus BX60 epifluorescence microscope using a FITC or Cy3-specific filter. All microscopic counts were determined in duplicate, with a minimum of 300 cells counted per assay.

Table 1 Genus-specific probes used for FISH analysis

Probe	Target group	Ref.
EUB 338	Total bacteria	[44]
Bac 303	*Bacteroides-Prevotella* group	[45,46]
Bif 164	*Bifidobacterium*	[12]
Erec 482	*Clostridium coccoides-Eubacterium rectale* group	[47]
Chis 150	*Clostridium histolyticum* group	[47]
Clit 135	*Clostridium lituseburense* group	[47]
Cld 73	*Clostridium difficile*	This study: CGCCGC TCTTTACCGAAGT
Fprau 645	*Faecalibacterium prausnitzii*	[46,48]
Lab 158	*Lactobacillus-Enterococcus* group	[49]

DNA extraction and polymerase chain reaction (PCR) amplification of fecal and duodenal brush samples

Brush and fecal samples were thawed on ice cooled water. DNA was extracted using the DNeasy Tissue kit (Qiagen, Venlo, The Netherlands) or the Fast DNA Spin kit (Qbiogene, Irvine, USA) from the brush and fecal samples respectively. The eluted DNA samples were stored at -20℃. The integrity of the isolated DNA was determined visually after electrophoresis on a 1.0% agarose gel containing ethidium bromide.

Real-time PCR

Quantification of *Lactobacilli* genera and species

specifically belonging to bifidobacteria was performed using a 5'nuclease (TaqMan) assay as described previously[13,14]. Briefly, a 20 μL PCR amplification mixture containing 10 μL TaqMan Fast Universal Master Mix (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands), optimized concentrations of primers and probes and 2.0 μL isolated DNA was prepared. The temperature profile for the amplification consisted of 20 s at 95℃ and 45 cycles of 1 s at 95℃ and 20 s at 60℃ (ABI 7900 HT Fast; Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). The percentages of the different bacteria were subsequently calculated as described by Liu *et al*[15,16].

Statistical analysis

FISH results with microbial numbers below the detection limit (8.3×10^5/g) were excluded from statistical analysis. Nonparametric FISH microbiota data were compared using Mann-Whitney tests or Kruskal-Wallis test for subgroup analysis. Independent samples *t*-test was used to compare differences in real-time PCR microbiota data between IBS patients and healthy subjects. One-way ANOVA with Bonferroni correction was used for analysis of microbiota in the IBS subgroups.

P-values less than 0.05 were considered statistically significant. All statistical analysis was performed using commercially available software (SPSS 12.0.1 for Microsoft Windows). Data are expressed as mean ± SE.

RESULTS

Characterization of the fecal microbiota of IBS patients and healthy subjects

The mean percentages of all bacterial groups measured are presented in Table 2. The results show that *F. prausnitzii*, *E. rectale*/*C. coccoides* and bifidobacteria are the most abundant groups in both IBS patients and healthy subjects. The levels of bifidobacteria were significantly lower ($P < 0.05$) in IBS patients (4.2% ± 1.3%) than in healthy subjects (8.3% ± 1.9%). No significant differences were observed between IBS-D, IBS-C and IBS-A subgroups. The *C. lituseburense* group was detected in significantly lower levels ($P < 0.01$) in IBS patients compared to healthy subjects; however, *C. lituseburense* reached the detection limit only in 14 healthy subjects and 18 IBS patients. The proportions of *Lactobacillus* spp, *C. coccoides*, *C. histolyticum*, *C. difficile*, *Bacteroides* and *F. prausnitzii* showed no differences between IBS patients and healthy subjects. This set of probes covered 44% and 32% of the total fecal microbiota in the healthy subjects and IBS patients, respectively. The low coverage is predominantly due to low counts in Bacteroides.

Characterization of the fecal bifidobacteria microbiota of IBS patients and healthy subjects

In healthy subjects, the proportion of bifidobacteria identified as *Bifidobacterium catenulatum* (19.31% ± 2.5%) was significantly ($P < 0.001$) higher compared to IBS patients (6.24% ± 0.6%). The low proportion of *B. catenulatum* was consistent in all IBS subgroups (Table 3, Figure 1A). The proportions of the other species (*Bifidobacterium adolescentis*, *Bifidobacterium bifidum* and *Bifidobacterium longum*) were not significantly different between healthy subjects, IBS patients and IBS subgroups. Low levels of *B. bifidum* were detected in fecal samples of all subjects as compared to the other *Bifidobacterium* species and as compared to the *B. bifidum* level in duodenal samples (Tables 3 and 4). The bifidobacterial species covered by these Q-PCR assays were only 43% and 29.5% of the total bifidobacteria population for healthy subjects and IBS patients, respectively.

Table 2 FISH analysis of the composition of the fecal microbiota of HS, IBS patients and IBS subgroups

Probe	HS	IBS	IBS-A	IBS-D	IBS-C
Fprau 645	12.0 ± 2.1	9.2 ± 0.80	10.6 ± 1.6	8.2 ± 1.4	8.6 ± 1.2
Erec 482	16.6 ± 5.4	11.7 ± 2.5	6.4 ± 1.3	7.1 ± 1.2	20.5 ± 5.8
Bif 164	8.3 ± 1.9	4.2 ± 1.3[a]	1.7 ± 0.63	7.9 ± 5.2	4.5 ± 0.94
Lab 158	4.7 ± 0.88	4.0 ± 0.78	2.0 ± 0.38	4.0 ± 1.7	6.0 ± 1.5
Chis 150	2.4 ± 0.43	2.1 ± 0.57	2.0 ± 0.61	1.6 ± 0.37	2.4 ± 1.4
Bac 303	1.5 ± 0.89	3.6 ± 1.5	0.41 ± 0.21	4.4 ± 3.3	5.7 ± 2.8
Cld73	0.88 ± 0.28	0.43 ± 0.09	0.61 ± 0.16	0.32 ± 0.15	0.21 ± 0.08
Clit 135	0.39 ± 0.10	0.09 ± 0.03[a]	0.06 ± 0.03	0.09 ± 0.04	0.11 ± 0.06
Sum	44.4 ± 6.85	32.1 ± 3.27	22.7 ± 2.30	28.3 ± 5.90	43.5 ± 5.89

[a]$P < 0.05$ *vs* HS. Data are expressed as percentage, mean ± SE.

Table 3 Real time PCR analysis of fecal bifidobacteria in HS, IBS patients and IBS subgroups

	HS	IBS	IBS-A	IBS-D	IBS-C
B. catenulatum	19.31 ± 2.5	6.24 ± 0.6[b]	6.57 ± 1.1[b]	5.67 ± 0.8[b]	6.49 ± 1.2[b]
B. adolescentis	17.05 ± 2.5	15.96 ± 1.6	16.86 ± 3.4	16.73 ± 1.8	14.06 ± 3.05
B. bifidum	2.1×10^{-4} ± 1.4×10^{-4}	9.1×10^{-4} ± 6.3×10^{-4}	3.3×10^{-4} ± 2.2×10^{-4}	1.9×10^{-3} ± 1.8×10^{-3}	4.5×10^{-4} ± 3.1×10^{-4}
B. longum	7.11 ± 1.4	7.30 ± 0.8	6.68 ± 1.4	8.71 ± 1.7	6.45 ± 1.33

[b]$P < 0.001$ *vs* HS. Data are expressed as percentage, mean ± SE.

Characterization of the duodenal microbiota of IBS patients and healthy subjects

In healthy subjects, *B. catenulatum* level as percentage of total bifidobacterial load (17.04% ± 2.3%) was significantly ($P < 0.001$) higher when compared to IBS patients (4.85% ± 0.5%). The significantly lower proportion of *B. catenulatum* was observed in all IBS subgroups (Table 4, Figure 1B). The levels of *B. adolescentis, B. bifidum* and *B. longum* as percentage of total bifidobacterial load were comparable between healthy subjects, IBS patients and IBS subgroups (Table 4). With the set of probes used, the total percentage of bifidobacteria of the bifidobacterial load which could be detected is 46% for healthy subjects and 31% for IBS patients.

Characterization of B. catenulatum in age-matched IBS patients and healthy subjects

Since the patients and healthy subjects were not matched, the age difference between the healthy subjects and IBS patients may be a confounding factor. In a subset of the subjects, 19 IBS patients (33 ± 2.8) matched for age

Table 4 Duodenal mucosa-associated bifidobacteria in HS, IBS patients and IBS subgroups

	HS	IBS	IBS-A	IBS-D	IBS-C
B. catenulatum	17.04 ± 2.3	4.85 ± 0.5[b]	5.26 ± 0.9[b]	4.26 ± 0.7[b]	5.04 ± 1.0[b]
B. adolescentis	15.68 ± 2.3	13.90 ± 1.4	14.66 ± 2.7	14.58 ± 1.7	12.21 ± 3.0
B. bifidum	6.09 ± 0.9	5.13 ± 0.8	5.36 ± 1.7	4.43 ± 1.1	5.68 ± 1.3
B. longum	6.88 ± 1.3	7.27 ± 0.8	5.84 ± 1.3	9.01 ± 1.6	6.88 ± 1.40

[b]$P < 0.001$ *vs* HS. Data are expressed as percentage, mean ± SE.

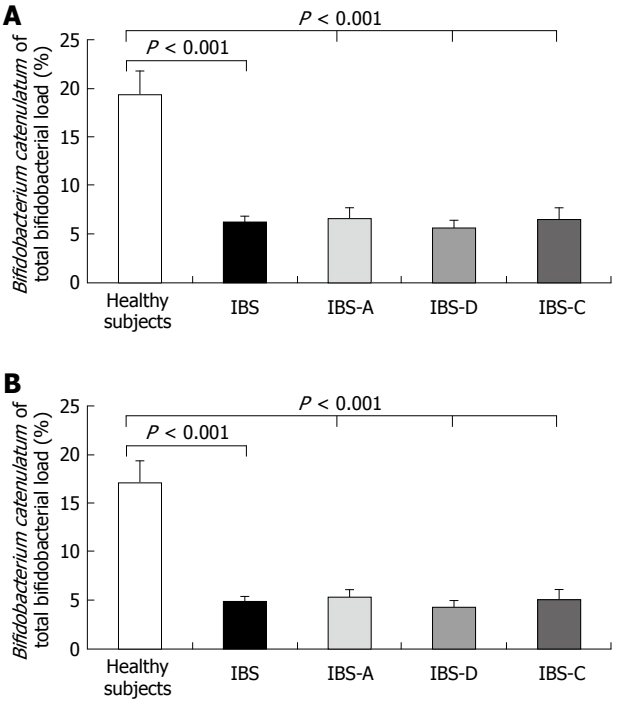

Figure 1 Percentage of *Bifidobacterium catenulatum* as percentage of total bifidobacterial load in fecal samples and duodenal samples of healthy subjects, IBS patients and IBS subgroups (mean ± SE). A: Fecal samples; B: Duodenal samples.

with 19 healthy subjects (33 ± 2.7), decreased levels of *B. catenulatum* were also shown in duodenal as well as in fecal samples of the IBS patients. The mean percentage of *B. catenulatum* of total bifidobacterial load in duodenal samples was significantly ($P < 0.001$) lower in IBS patients (5.48% ± 0.60%) compared to healthy subjects (17.19% ± 2.43%). Percentage of *B. catenulatum* of total bifidobacterial load in the fecal samples was significantly ($P < 0.001$) lower in IBS patients (6.98% ± 0.69%) compared to healthy subjects (19.50% ± 2.67%).

DISCUSSION

Composition of gastrointestinal microbiota is known to be relatively stable and composed of permanent, the so-called core phyla, and transient species which contribute to gastrointestinal health and disease[17-19]. The presence of beneficial microbes in the intestine prevents colonization by potentially pathogenic microbes, referred to as colonization resistance[20,21]. Imbalances in the microbiota are characterized by a decrease in beneficial anaerobic bacteria and increases in aerobic bacteria, fungi and harmful anaerobic bacteria[20].

In this study, we showed, using FISH analysis, that IBS patients have significantly lower fecal levels of bifidobacteria but no differences in the other major bacterial groups. Previous studies have also shown microbial alterations in fecal samples of IBS patients using both culturing and molecular-based techniques[2,4]. Using culturing techniques, Balsari *et al*[2] have already shown in 1982 a decrease in bifidobacteria, coliforms and lactobacilli in IBS patients. Using molecular-based techniques decreased *B. catenulatum*, *C. coccoides*, *Lactobacillus* and *Collinsella* counts in the fecal samples of IBS patients were found[4,5]. These studies were limited to the fecal flora. We broadened the study by examining bifidobacteria levels in duodenal mucosa-associated samples.

Differences in microbiotic composition between luminal and mucosa-associated bacteria have been shown[7,22-25]. The different micro-environment of the epithelium compared to the lumen might lead to a different microbiotic composition[7,26]. Bacteria that attach to the mucosa may exert greater influence on innate immune processes in the intestine[24,27]. In addition, the adhesion of non-pathogenic bacteria to the epithelial surface may contribute to the barrier that effects host resistance to pathogenic bacteria[24].

In this study, we showed that the percentages of *B. bifidum* of the total bifidobacterial counts were lower in the fecal samples than in the duodenal mucosa-associated samples in both IBS patients and controls. This might be due to high hydrophobicity of *B. bifidum* which is related to its ability to adhere to surfaces[28,29]. Furthermore, *B. catenulatum* counts were decreased in duodenal mucosa-associated samples as well as in fecal samples of IBS patients compared to controls. The effect of *B. catenulatum* on the health of the host is unknown. However as a group, bifidobacteria are considered beneficial for the host, as they produce lactic and acetic acids that decrease pH and inhibit the growth of potential pathogenic bacteria[30-32].

Moreover, *Bifidobacterium* spp prevent diarrhea and intestinal infections, alleviate constipation and stimulate the immune system[31]. The lower levels of bifidobacteria might be epiphenomenal or develop as a consequence of altered gastrointestinal motility or genetic makeup of IBS patients rather than being the cause of IBS symptoms[31].

Since the patients and healthy subjects were not matched, the age difference between the healthy subjects and IBS patients might have been a confounding factor. It was reported that elderly (> 65 years old) have lower fecal levels of *B. catenulatum*[33,34]. The elderly were not included in our study. The effect of the age difference between healthy subjects (mean age 32 years) and IBS patients (mean age 42 years) on *B. catenulatum* levels is not known. However, in age-matched IBS patients and healthy subjects statistically significantly decreased levels of *B. catenulatum* were also seen in duodenal as well as in fecal samples of the IBS patients.

An imbalanced microbiotic composition may lead to a different fermentation pattern, especially with an increased hydrogen production resulting in bloating[6,35].

Both antibiotics and probiotics have been shown to reduce IBS symptoms, which further suggests that microbial imbalance may underlie symptom generation in IBS patients[36-40]. Previously, a therapeutic trial suggested that particular *B. infantis* species were efficacious in the treatment of IBS symptoms[41]. No effects of *B. infantis* on stool consistency or frequency could be observed which implies that this therapeutic approach may be applicable to all IBS patients irrespective of their stool pattern[41]. *B. breve* in combination with *L. plantarum* has been shown to decrease pain and the severity of symptoms in IBS patients[42]. Prebiotics, oligofructose and inulin might reduce symptoms in IBS-C patients as they selectively stimulate bifidobacteria which results in increased stool frequency[43].

In conclusion, lower bifidobacteria levels were found both in duodenal mucosa-associated samples as well as in fecal samples of IBS patients when compared to healthy subjects. Specifically, *B. catenulatum* was found to be reduced in duodenal mucosa-associated bacteria as well as in the feces of IBS patients. The relevance of specific *Bifidobacterium* spp in relation to IBS symptoms is unknown; however modulation of the gut microbiota by means of prebiotics or bifidobacteria-containing probiotics to restore a balanced microbiotic composition may have a therapeutic role.

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COMMENTS

Background

Alterations in fecal and small intestinal microbiotic composition in irritable bowel syndrome (IBS) patients have been reported and studies revealed a somewhat higher bacterial count in jejunal juice of IBS patients and lower numbers of fecal coliforms, lactobacilli and bifidobacteria than in healthy subjects.

Research frontiers

The aim of the study was to determine the composition of both fecal and duodenal mucosa-associated microbiota in IBS patients and healthy subjects using molecular-based techniques.

Innovations and breakthroughs

Decreased bifidobacteria levels in both fecal and duodenal brush samples of IBS patients compared to healthy subjects indicate a role for microbiotic composition in IBS pathophysiology.

Peer review

The authors investigated the fecal and duodenal mucosal-associated microbiota in patients with IBS. This topic is of interest since, as the authors mentioned, alterations in intestinal flora have been suggested as a potential etiological factor in the pathogenesis of this disorder.

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Glutamine synthetase activity and glutamate uptake in hippocampus and frontal cortex in portal hypertensive rats

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Abstract

AIM: To study glutamine synthetase (GS) activity and glutamate uptake in the hippocampus and frontal cortex (FC) from rats with prehepatic portal vein hypertension.

METHODS: Male Wistar rats were divided into sham-operated group and a portal hypertension (PH) group with a regulated stricture of the portal vein. Animals were sacrificed by decapitation 14 d after portal vein stricture. GS activity was determined in the hippocampus and FC. Specific uptake of radiolabeled L-glutamate was studied using synaptosome-enriched fractions that were freshly prepared from both brain areas.

RESULTS: We observed that the activity of GS increased in the hippocampus of PH rats, as compared to control animals, and decreased in the FC. A significant decrease in glutamate uptake was found in both brain areas, and was more marked in the hippocampus. The decrease in glutamate uptake might have been caused by a deficient transport function, significantly and persistent increase in this excitatory neurotransmitter activity.

CONCLUSION: The presence of moderate ammonia blood levels may add to the toxicity of excitotoxic glutamate in the brain, which causes alterations in brain function. Portal vein stricture that causes portal hypertension modifies the normal function in some brain regions.

Key words: Portal hypertension; Glutamine synthetase; Glutamate uptake; Frontal cerebral cortex; Hippocampus; Rat

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INTRODUCTION

Two major complications appear in severe liver failure: hepatic encephalopathy (HE) and portal hypertension (PH), but the mechanism involved in the production of brain damage is still unclear.

PH is found in patients with cirrhosis, and in portal vein thrombosis, it is characterized by an increase in splanchnic blood flow and pressure, caused by abdominal blood flow resistance, secondary to important liver parenchyma alterations (fibrosis or cirrhosis). Normally, the liver splanchnic blood must reach, through the liver, the hepatic veins and finally the vena cava[1]. As a result

of increased splanchnic blood flow, collateral vein shunts appear and abdominal circulation avoids the damaged liver parenchyma to reach the systemic circulation[2].

HE in acute and chronic liver disease is a complex syndrome, associated frequently with hyperammonemia. Increases in blood brain barrier (BBB) permeability present in PH, allow ammonia ions and other neurotoxic substances to penetrate brain tissue[3-6]. Hyperammonemia is caused by a defect in the liver parenchyma that forms urea from intestinal nitrogenic substances, and vein shunts from the splanchnic circulation carry it into the general circulation[7].

According to Erceg *et al*[8], chronic hyperammonemia, with or without liver failure, impairs the glutamate-nitric oxide-cGMP pathway in the brain and reduces extracellular cGMP in the brain. This function is associated with a decreased ability of rats to learn a Y maze conditional discrimination task. It has been suggested that a decrease in extracellular cGMP is involved in impaired learning ability and intellectual function.

Ammonia is a well-known toxic substance for the central nervous system (CNS), especially when levels exceed the antitoxic capacity of the brain cells. Glutamate plays an important role in cellular metabolism, and contributes to normal excitatory neurotransmission in the brain. When this function is not accomplished effectively, and either ammonia or glutamate are not sufficiently detoxified, their concentrations increase pathologically, neuron and astrocyte functions deteriorate, and damage and even cell death can result[9]. It has been shown clearly that acute ammonia toxicity and liver failure lead to excitotoxicity as a result of activation of N-methyl-D-aspartate (NMDA) receptors in the brain[10], and that blocking these receptors can lead to ammonia-induced death[11]. In contrast, chronic hyperammonemia leads to down-regulation of signal transduction pathways associated with these receptors, which contributes to cognitive impairment[8].

The glutamine/glutamate cycle participates in cell metabolism, and has important relevance in normal and pathological functions. When this cycle does not function adequately, CNS functional damage can appear, and even cellular death can be produced[12]. To accomplish the transformation to ammonia and glutamate into glutamine, the brain depends on the activity of the enzyme glutamine synthetase (GS) in astrocytes. This is associated with correct function of the glutamate transporters, to provide an adequate uptake, release and metabolism of ammonia and glutamate[13]. Therefore, the importance of correct function of the glutamine/glutamate cycle during this detoxifying step in brain is clear.

The aim of the present study was to analyze the participation of GS activity and glutamate uptake in the hippocampus and cerebral frontal cortex (FC), using a prehepatic PH rat model, with the intention to mimic the two major complications that appear in chronic liver failure. By using this model, it may be possible to understand more clearly the defense mechanism of the brain against the two toxic substances, ammonia and the excitatory neurotransmitter glutamate.

MATERIALS AND METHODS

Animals and surgical procedures

Male Wistar rats with an average weight of 240 g were used. The animals were placed in individual cages, with free access to food (standard laboratory rat chow) and water, and a 12-h light cycle: 8.00-20.00 h. Special care for perfect air renewal was taken. PH was obtained by calibrated stenosis of the portal vein, according to the method of Chojkier and Groszmann[14]. Rats were lightly anesthetized with ether and then a midline abdominal incision was made. The portal vein was located and isolated from the surrounding tissues. A ligature of 3.0 silk sutures was placed around the vein, and snugly tied to a 20-gauge blunt-end needle placed alongside the portal vein. The needle was subsequently removed to yield a calibrated stenosis of the portal vein. Fourteen days after portal vein ligation, animals exhibit an increase in portal pressure. Sham-operated rats underwent the same experimental procedure, except that the portal vein was isolated but not stenosed. Animals were placed in individual cages and allowed to recover from surgery. Animals were sacrificed by decapitation at 14 d after portal vein stricture.

Experiments were carried out in accordance with the US National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publication Nº 80-23/96) and local regulations. All efforts were made to minimize suffering of animals and to reduce the number of animals used.

Portal pressure measurement

Fourteen days after the corresponding operation, the rats were anesthetized with intraperitoneal sodium pentobarbital (40 mg/kg). Portal pressure was measured through a needle placed in the splenic pulp, and maintained in place by cyanoacrylate gel. The needle was cannulated to a polyethylene catheter (50) filled with a heparinized saline solution (25 U/mL), and connected to a Statham Gould P23ID pressure transducer (Statham, Hato Rey, Puerto Rico), coupled to a Grass 79D polygraph (Grass Instruments, Quincy, MA, USA).

GS activity

GS activity was assessed as described by Rowe *et al*[15], with some minor modifications. Fourteen days after portal vein stricture, rats were sacrificed by decapitation and the FC and hippocampus were incubated in 500 μL HEPES-Tris buffer, which contained 140 mmol/L NaCl, 5 mmol/L KCl, 2.5 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, 10 mmol/L HEPES, 10 mmol/L glucose (adjusted to pH 7.4 with Tris base) for 30 min at 37℃. After incubation, each brain region was homogenized in 200 μL 10 mmol/L potassium phosphate, pH 7.2. Reaction mixtures contained 150 μL brain region homogenate and 150 μL stock solution (100 mmol/L imidazole-HCl buffer, 40 mmol/L $MgCl_2$, 50 mmol/L β-mercaptoethanol, 20 mmol/L ATP, 100 mmol/L glutamate and 200 mmol/L hydroxylamine, adjusted to pH 7.2) Tubes were incubated at 37℃ for 15 min.

The reaction was stopped by adding 0.6 mL ferric chloride reagent (0.37 mol/L $FeCl_3$, 0.67 mol/L HCl and 0.20 mol/L trichloroacetic acid). Samples were placed for 5 min on ice. Precipitated proteins were removed by centrifugation at 10000 *g*, and the absorbance of the supernatants was read at 535 nm against a reagent blank. Under these conditions, 1 μmol γ-glutamylhydroxamic acid gave an absorbance of 0.340. GS specific activity was expressed as μmol γ-glutamylhydroxamate per hour per milligram of protein.

Preparation of tissue samples for glutamate uptake

As described for the GS activity assay, at 14 d after portal vein stricture, animals were killed by decapitation. The brain was removed from the cranial cavity, and the FC and hippocampus were dissected onto a Petri dish at 0℃, according to the method of Glowinski and Iversen[16], and homogenized with a glass-PTFE homogenizer in 15 volumes of 0.32 mol/L sucrose. The homogenates were centrifuged at 800 *g* for 10 min, the pellet was discarded, and the supernatant was centrifuged at 20000 *g* for 20 min. The pellet (P2 = crude synaptosomal fraction) was suspended with a glass-PTFE homogenizer in fresh 0.32 mol/L sucrose, and again centrifuged at 20000 *g* for 20 min. The procedure was repeated three times, and the resulting pellet was suspended and used in uptake experiments within 5 h after preparation.

Glutamate uptake procedure

Uptake experiments were carried out using fresh synaptosomal fractions that originated from 20 mg of tissue of FC and hippocampus (wet weight) per 1 mL incubation medium. This consisted of 125.0 mmol/L NaCl, 3.5 mmol/L KCl, 1.5 mmol/L $CaCl_2$, 1.2 mmol/L $MgSO_4$, 1.25 mmol/L KH_2PO_4, 25 mmol/L $NaHCO_3$, 10 mmol/L HEPES and 10 mmol/L D-glucose, pH adjusted to 7.4. The tissue was first incubated for 5 min at 30℃, as described by Takarada *et al*[17], followed by the addition of 10 nmol/L radiolabeled substrate of [^{3}H] L-glutamate, and subsequent incubation for 5-30 min (time course study). The incubation was terminated by vacuum-filtration through Whatman glass fiber-filters (type D) and rapid washing, three times, with isotonic saline solution (at 2-4℃). The radioactivity on the filters was measured using liquid scintillation counting. Parallel experiments were always performed without incubation as time zero, to obtain radioactivity not specifically taken up into brain preparation for the radiolabeled substrate used in this experiment.

Protein content was estimated by the technique of Lowry *et al*[18] using bovine serum albumin as a standard.

Drugs, chemicals and radiolabeled compounds

[^{3}H] L-Glutamate (specific activity: 52.0 Ci/mmol) was from Perkin Elmer NEN Life Science Inc. (Boston, MA, USA). Plasma ammonia concentration was determinate by the Ammoniac Enzymatic UV kit (Biomerieux, France). All other chemicals and reagents were analytical grade and obtained through regular commercial sources.

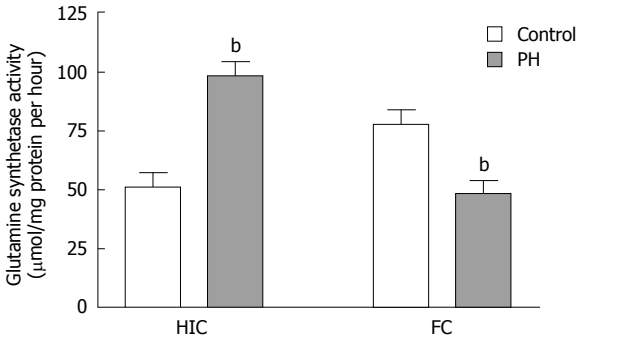

Figure 1 GS activity in the hippocampus and FC. Values are the mean ± SE in eight experiments. $^bP < 0.01$ compared with respective control group. HIC: Hippocampus.

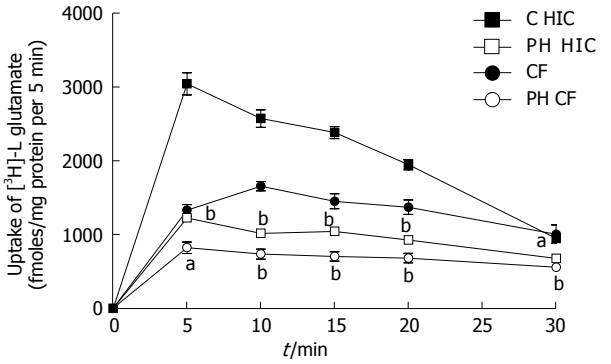

Figure 2 Time course of [^{3}H] L-glutamate uptake in FC and hippocampus. Synaptosomes were incubated with [^{3}H] L-glutamate at 10 nmol/L substrate concentration, for > 30 min, in the presence of 125 mmol/L NaCl at 30℃. Values are the mean ± SE in six or seven separate experiments done in triplicate. $^aP < 0.05$; $^bP < 0.01$ compared with respective control group. C: Control group; PH: Portal hypertension group.

Statistical analysis

The uptake was estimated with Graph Pad Prism 3.1 software (San Diego, CA, USA). Data of uptake activity were presented as the mean ± SE, and were analyzed by one-way analysis of variance, followed by Tukey test[19]. Student's *t* test was used to assess differences between paired groups. $P < 0.05$ was considered as significant.

RESULTS

In these experiments using prehepatic portal hypertensive animals, with almost normal liver function (2-2.5 times normal values), we found a significant increase in hippocampal GS activity, as compared to sham-operated animals ($P < 0.001$) (Figure 1). On the contrary, in FC, a significant decrease in the enzyme activity was documented ($P < 0.001$), in comparison with control rat brains (Figure 1).

The uptake of [^{3}H] glutamate occurred in a temperature-dependent manner and increased with incubation time up to 5 min, with a plateau thereafter at 30 min. Glutamate uptake in the hippocampus and FC showed that, at 30℃, [^{3}H] L-glutamate was taken up into synaptosomes in a time-dependent manner. Its level reached a plateau after 20 min in the presence of Na^+ ions (Figure 2), and remained linear with time for about 2-3 min in the hippocampus and FC, in PH and

sham-operated rats. The uptake of [^{3}H] L-glutamate was temperature- and Na^+-dependent in both regions studied. Time course experiments verified that the uptake of this excitatory amino acid was essentially linear up to 2-3 min at the respective incubation temperature (Figure 2).

The portal hypertensive rat, with almost normal liver function (only moderately increased aspartate aminotransferase and alanine aminotransferase activity and blood ammonia), was shown to have morphological and functional alterations of GS and glutamate uptake in astrocytes and neurons.

There was a significant decrease in glutamate uptake in the portal hypertensive group, as compared to the sham-operated rats, in the hippocampus ($P < 0.001$) and FC ($P < 0.05$) (Figure 2). We found a more pronounced decrease in glutamate uptake in the hippocampus. [^{3}H] L-Glutamate uptake was concentration-dependent at 30℃ and was dramatically suppressed at 2℃ (data not shown). At 30 min, we observed a decline in [^{3}H] L-glutamate uptake in the two regions, which might have been caused by the metabolic changes in the glutamine/glutamate cycle.

Liver homogenates obtained from both groups of animals showed no biochemical alterations and no histological damage (data not shown).

Portal pressure was 8.5 ± 0.5 mmHg in the sham-operated group and 12.5 ± 0.8 mmHg in the PH group ($P < 0.01$).

Plasma ammonium level was 23 ± 9 μmol/L in the control group and 79 ± 15 μmol/L in the PH group, which corresponded to an increase of 243% ($P < 0.01$).

DISCUSSION

PH is responsible for severe circulatory derangements in the splanchnic and systemic circulation, and brain damage results from HE, as a consequence of acute and chronic liver failure. In particular, HE is responsible for severe and often lethal outcomes in these diseases. The HE can be characterized by a wide spectrum of neuropsychiatric abnormalities.

In the present study of prehepatic portal hypertensive rats, we demonstrated different activities of GS and glutamate uptake in the hippocampus and FC. We observed that the activity of GS increased in the hippocampus of PH rats compared to control animals, while there was a decrease in the FC. There was a moderate increase in ammonia concentration.

Using fresh synaptosomal fractions from the FC and hippocampus, we found that glutamate uptake was decreased significantly in PH rats, with a more marked decrease in the hippocampus. In the presence of 125 mmol/L NaCl, uptake of this radiolabeled amino acid occurred in a temperature-dependent manner, and increased with incubation time up to 2-3 min, with a plateau thereafter at 30 min. These results suggest that there are biochemical differences between the brain regions, possibly caused by the toxic metabolic action of ammonia, glutamate, and perhaps glutamine, on the rat brain.

The morphological alterations in the liver parenchyma in chronic disease and in portal vein thrombosis create collateral veins that shunt splanchnic blood flow to the systemic circulation, in an attempt to overcome the increased pressure of portal vein flow. This phenomenon modifies the normal physiology of several organs, including the CNS, and transports intestinal toxins directly to the brain.

In previous experiments with this rat model, alterations in the CNS have been documented, including BBB permeability modifications[5,20]. The impact of PH on the BBB has been demonstrated in rats, in which, 40 d after portal vein stricture, the BBB recovers its function associated to normalize portal pressure and the impermeability to dyes[20].

Glutamate is a major excitatory neurotransmitter, and any alteration in the glutaminergic pathway must modify brain function. Normally, glutamate is synthesized in brain tissue from glucose.

Butterworth *et al*[21], studied the brain in liver failure, glutamate, some other amino acids and neurotransmitters, such as serotonin and dopamine, that are involved in the development of HE. Glutamate uptake and transport are important steps in protecting CNS cells from glutamate excitotoxicity[22,23].

As the removal of ammonia in the brain is linked to the metabolism of glutamate in astrocytes, damage to these cells has been described in PH animals, which involves glutamate uptake and clearance[24].

Rapid clearance of neurotransmitters released from synapses, especially glutamate, acts to limit its signaling and prevents its harmful over-stimulation[25]. It has been well established for several decades that hyperammonemia leads to reduced glutamate uptake, which is caused by a reduced amount and function of glutamate transporters[26-28]. Moreover, it has been shown recently that reduced glutamate transporters and increased extracellular glutamate are responsible for hypokinesia in rats with HE and hyperammonemia[29].

GS plays a central role in the metabolic regulation of the excitatory neurotransmitter glutamate and in the detoxification of ammonia. GS is located mainly in astrocytes. It has been suggested that glutamate can regulate GS brain distribution[30]. This enzyme is responsible for the protection of neurons against excess ammonia and glutamate, by metabolizing both substances into glutamine. Approximately 85% of ammonia is converted to glutamine[31,32]. In addition, glutamate from neurons can be reconverted into glutamine[33]. Astrocytes play a key role in the pathogenesis of ammonia-induced neurotoxicity and HE. Schliess *et al*[34] have found that ammonia induces protein tyrosine nitration in cultured rat astrocytes, which is sensitive to the NMDA receptor antagonist MK-801. Actually, the production of reactive nitrogen intermediates and protein tyrosine nitration may alter astrocyte function and contribute to ammonia neurotoxicity[34].

Acute intoxication with large doses of ammonia leads to CNS cell damage. The main mechanism for ammonia elimination in the brain is its reaction with glutamate to form glutamine. This reaction is catalyzed by GS and consumes ATP[35]. It has observed that GS activity and

glutamine content in the brain are modulated by NMDA receptors and nitric oxide.

There are two main types of hyperammonemia: (1) chronic moderate hyperammonemia, as occurs in liver cirrhosis, which leads to altered cerebral function, and is responsible for the neurological alterations in different hyperammonemic states, and also for some of the neurological alterations in liver disease and HE; and (2) acute intoxication with large doses of ammonia, which may lead to rapid death of animals or patients. This situation can occur in acute liver failure.

Direct toxic effects of glutamine on CNS cells have been demonstrated in isolated mitochondria, which shows that elevated accumulation of glutamine has injurious effects on these cells[36].

Isolated rat cerebral mitochondria treated with high glutamine concentrations (5 mmol/L), as present in acute hyperammonemic rats, show swelling and mitochondrial permeability transition (MPT)[37]. Murthy *et al*[38] have shown that ammonia alone does not induce MPT, but that its metabolism to glutamine is necessary to produce this alteration. Mitochondrial swelling, as a result of the presence of high levels of glutamine and ammonia, is known to stimulate the uptake of glutamine[39].

The addition of glutamine to cultured astrocytes induces MPT development and the formation of free radicals[40]. Hence, glutamine synthesis, from ammonia and glutamate in astrocytes, represents an important process in brain ammonia detoxification, but also can produce negative effects.

The increased activity of GS in the FC, as seen in this PH rat model, may represent a response to moderate increases in ammonia. However, in the hippocampus, we observed decreased activity of GS, which may correspond to an increase in glutamate, caused by decreased uptake and a prolonged toxic effect.

The astrocyte is the brain cell that is directly involved in HE. It participates in chronic porto-systemic encephalopathy, with ultrastructural alterations in the brain. Astrocyte mitochondria are included in this pathophysiological mechanism in the hippocampus[41] and alterations in its respiratory chain, and show ultrastructural damage. By using proton magnetic resonance, Häussinger[42] has shown that brains from HE patients have an increase in glutamine/glutamate signaling. Structural and functional alterations in cultured astrocytes, when their exposure to different ammonia concentrations is modified, suggest the possibility that HE may constitute a primary gliopathy[43].

The mechanisms that produce brain damage in chronic encephalopathy may include the following. (1) Increased ammonia in the brain, which is associated with an increase in glutamate, caused by its reduced uptake, which leads to altered metabolic pathways and functional and morphological damage to the mitochondria in the hippocampus and FC; and (2) The hippocampus possesses a fundamental function in behavioral patterns on memory and spatial mechanisms among other functions. Therefore, it is possible that hippocampus suffers more readily from toxicity than the FC, which also participates in this process, but with minor involvement.

It is possible that the results obtained in the hippocampus in PH rats are indicative of its plasticity, which provokes, during toxic insult, changes in the expression of its enzymes, which maintain the functional equilibrium during this pathological situation. In the mammalian brain, this region is fundamental for the encoding of recent and past experience, including spatial and non-spatial information[41]. The FC has an efficient adaptative mechanism, not described in the hippocampus; its structure perhaps suffers more damage when toxic substances, such as ammonia, glutamate and glutamine are not detoxified efficiently. It may be that, in patients with cirrhosis, these mechanisms can participate in memory processes and behavioral alterations. It might be that the extracellular glutamate that arises from impairment of the glutamate/glutamine cycle in PH participates in the pathogenesis of HE. Furthermore, in PH, increases in ammonia and glutamate and/or glutamine may participate in oxidative stress that is induced by a glutamate-mediated pathway.

Warren and Schenker[44] have demonstrated that inhibition of GS by methionine sulfoximine decreases the death rate in rats intoxicated with ammonia. They found fewer metabolic alterations in HE, including brain edema, less astrocyte swelling, and a decrease in ammonia-induced reactive oxygen species. These experiments demonstrate the negative role that glutamine can play in the pathophysiology of HE[39,45-47]. In liver failure, brain edema, intracranial hypertension, neurotransmitter derangements and neurological symptoms represent the cerebral repercussions of distant liver parenchymal toxicity.

Our results from a PH rat model showed that two different brain regions had different responses in terms of GS activity and glutamate uptake. The BBB in these animals showed an increase in its permeability, apparently as a result of increased portal pressure, because when pressure returned to normal, the BBB recovered its permeability[20]. Also, an increase in astrocyte number, associated with an increase in endothelial cells (angiogenesis), has been documented previously[48].

Finally, it is clear that a partial stricture of the portal vein is capable of modifying functions in two brain regions, the hippocampus and FC. The different changes observed are difficult to explain. Perhaps, further experiments with PH models, using blockers and antagonist of glutamate uptake are needed to explain these results.

COMMENTS

Background

To analyze the participation of glutamine synthetase (GS) activity and glutamate uptake in the hippocampus and cerebral frontal cortex (FC), using a rat model of prehepatic portal hypertension (PH), with the intention of mimicking the two major complications that appear in chronic liver failure: hepatic encephalopathy (HE) and PH.

Research frontiers

In this field, the research hotspots are how to use prehepatic PH rats and how to study the different activities of GS and glutamate uptake in the hippocampus and FC.

Innovations and breakthroughs

This study determinate the uptake of [^{3}H] L-glutamate by synaptosomes of rat cerebral cortex. That provides accurate and reproducible data and can be employed to measure kinetic parameters, and this study determinate GS activity to maintain a stable glutamate/glutamine ratio in the brain.

Applications

Using the PH model, it is possible to understand more clearly the mechanism of toxicity and defense of the brain against two toxic substances: ammonia and the excitatory neurotransmitter glutamate.

Terminology

The extracellular actions of glutamate are limited by glutamate-specific Na^+-dependent transporters that remove glutamate, mostly by taking it up into astrocytes. Astrocytes convert glutamate into glutamine and pass it back to neurons, where it can be converted into glutamate and used to replenish the neurotransmitter stores in synaptic vesicles.

Peer review

The authors have used a rat model of PH and have analyzed the activity of GS and the uptake of glutamate by synaptosomes in the hippocampus and FC 14 d after surgery. The paper seems to be important for understanding HE. The whole paper is relatively easy to read and understand.

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BRIEF ARTICLES

Diagnostic value of maximal-outer-diameter and maximal-mural-thickness in use of ultrasound for acute appendicitis in children

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Abstract

AIM: To evaluate the maximal-outer-diameter (MOD) and the maximal-mural-thickness (MMT) of the appendix in children with acute appendicitis and to determine their optimal cut-off values to diagnose acute appendicitis.

METHODS: In total, 164 appendixes from 160 children between 1 and 17 years old (84 males, 76 females; mean age, 7.38 years) were examined by high-resolution abdominal ultrasound for acute abdominal pain and the suspicion of acute appendicitis. We measured the MOD and the MMT at the thickest point of the appendix. Patients were categorized into two groups according to their medical records: patients who had surgery (surgical appendix group) and patients who did not have surgery (non-surgical appendix group). Data were analyzed by MedCalc v.9.3. The rank sum test (Mann-Whitney test) was used to evaluate the difference in the MOD and the MMT between the two groups. ROC curve analysis was used to determine the optimal cut-off value of the MOD and the MMT on diagnosis of acute appendicitis.

RESULTS: There were 121 appendixes (73.8%) in the non-surgical appendix group and 43 appendixes (26.2%) in the surgical appendix group. The median MOD differed significantly between the two groups (0.37 cm *vs* 0.76 cm, $P < 0.0001$), and the median MMT also differed (0.15 cm *vs* 0.33 cm, $P < 0.0001$). The optimal cut-off value of the MOD and the MMT for diagnosis of acute appendicitis in children was > 0.57 cm (sensitivity 95.4%, specificity 93.4%) and > 0.22 cm (sensitivity 90.7%, specificity 79.3%), respectively.

CONCLUSION: The MOD and the MMT are reliable criteria to diagnose acute appendicitis in children. An MOD > 0.57 cm and an MMT > 0.22 cm are the optimal criteria.

Key words: Appendicitis; Ultrasonography; Pediatrics; Diagnosis; ROC curve

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INTRODUCTION

Since Puylaert[1] described the role of ultrasound (US) as a diagnostic method for acute appendicitis in 1986, the diagnosis of acute appendicitis has become more dependent on the use of US, especially in the doubtful cases for the clinicians. Because of the smaller trunks and thinner subcutaneous fat layer in children compared to adults, US evaluation of the appendix is easier in children. According to previous reports[1-4], radiologists have used several US findings to diagnose acute appendicitis. Among these findings, the maximal outer diameter (MOD) of the appendix was regarded as the most reliable measurement. When the MOD is > 0.6 cm, radiologists suggest the presence of acute appendicitis is indicated.

However, the MOD may be larger than 0.6 cm without acute inflammation. The concern is that the

MOD may be exaggerated by the presence of intra-luminal materials such as gas, feces and fluid[5-7]. To decrease the false positive rate of the MOD criterion, some radiologists have recently attempted to determine another size criterion, the maximal mural thickness (MMT) of the appendix[8-10].

The purpose of this study was to evaluate the diagnostic value of the MOD and the MMT measurements of the appendix in children with clinical suspicion of acute appendicitis and to determine the optimal cut-off values of these measurements in diagnosis of acute appendicitis.

MATERIALS AND METHODS

Among the abdominal US of the children who visited our institute for acute abdominal pain and the suspicion of acute appendicitis between July 2004 and November 2008, we selected 160 children who had a visible appendix on US. These children were aged 1-17 years (84 males, 76 females; mean age, 7.38 years).

After receiving informed consent, the children were examined by experienced radiologists with three different US units (iU22, Philips Medical Systems, Andover, MA, USA; HDI 5000 SonoCT, Philips Medical Systems, Best, The Netherlands; ATL HDI 5000, Philips Medical Systems, Andover, MA, USA). The appendix was scanned from the base to the tip under graded compression using a high resolution transducer (9-12 MHz linear transducer or a 5-8 MHz sector transducer). Then the MOD and the MMT were measured at the thickest point in the cross-sectional image (Figure 1). The MOD was defined as the distance between the outer hyperechoic borders of the the appendix, and the MMT was defined as the distance from the hyperechoic luminal interface to the outer hyperechoic border. Intra-luminal contents including fluid, gas, feces, stones or nothing were recorded.

The medical records of the patients were traced till the symptoms were resolved. We categorized the patients into two groups: patients who had surgery (surgical appendix group) and patients who recovered without surgery (non-surgical appendix group). Data were analyzed by MedCalc v.9.3 software (MedCalc, Mariakerke, Belgium). We used the rank sum test (Mann-Whitney test) to evaluate the difference in the MOD and the MMT between the two groups and used ROC curve analysis to determine the optimal cut-off values of the MOD and the MMT for diagnosing acute appendicitis.

RESULTS

The MOD and the MMT of 164 appendixes in 160 children were included in this study. Forty-four children underwent an appendectomy. The pathological diagnoses were 15 cases of acute appendicitis (without any comment), six cases of acute early appendicitis, 13 cases of acute suppurative appendicitis, six cases of acute gangrenous appendicitis, two cases of acute gangrenous appendicitis with perforation, one case of acute necrotizing appendicitis and one case of congestion. As congestion did not contain any inflammatory cells, the case of congestion was re-classified in the non-surgical appendix group. The case of congestion was finally diagnosed with Crohn's disease in the ileum and cecum. As a result, there were 121 appendixes (73.8%) in the non-surgical appendix group and 43 appendixes (26.2%) in the surgical appendix group.

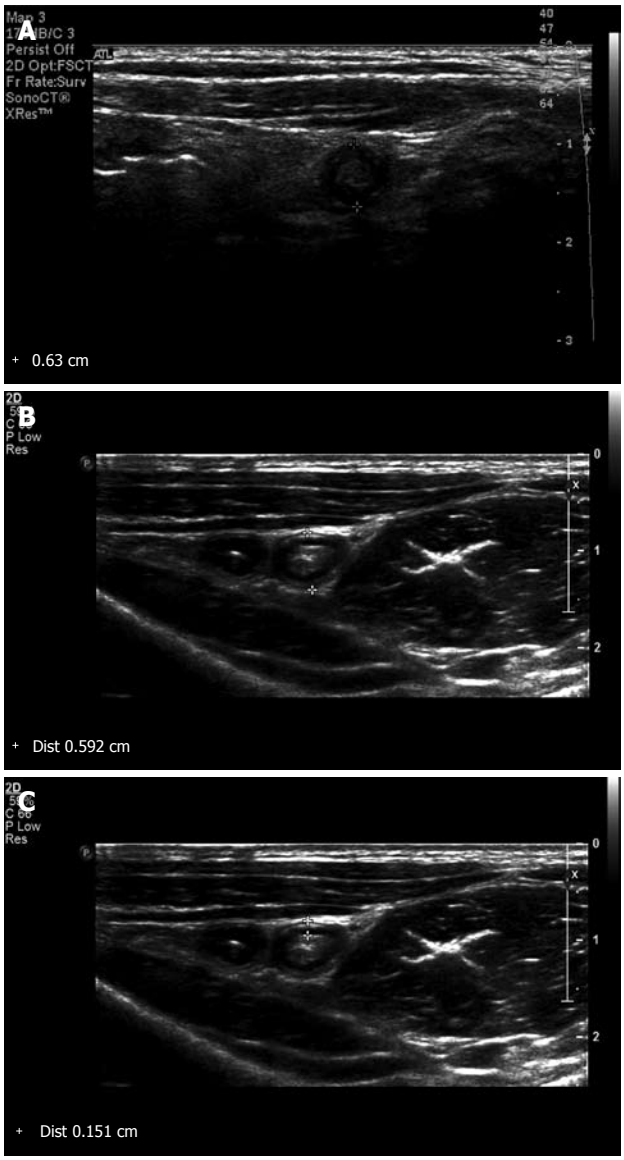

Figure 1 Measurement of MOD and MMT of an appendix in the cross-sectional image. The MOD (A) of the appendix of a 10-year-old boy who had surgery for acute appendicitis. The MOD (B) and the MMT (C) of the appendix of a 7-year-old boy who had a normal appendix in spite of acute abdominal pain.

Because each reference interval of the MOD and the MMT rejected normality ($P < 0.0001$ in each case), the Mann-Whitney test was used for the statistical analysis.

The range of the MOD was 0.20-1.45 cm in all patients, 0.20-0.69 cm in the non-surgical appendix group and 0.42-1.45 cm in the surgical appendix group. The median MOD of 0.37 cm (95% CI: 0.29-0.45 cm) in the non-surgical appendix group was significantly different ($P < 0.0001$) from the median MOD of 0.76 cm (95% CI: 0.69-0.90 cm) in the surgical appendix group. The data comparison graphs between the two groups were presented in Figure 2A.

The range of the MMT was 0.08-0.58 cm in all patients, 0.08-0.49 cm in the non-surgical appendix group

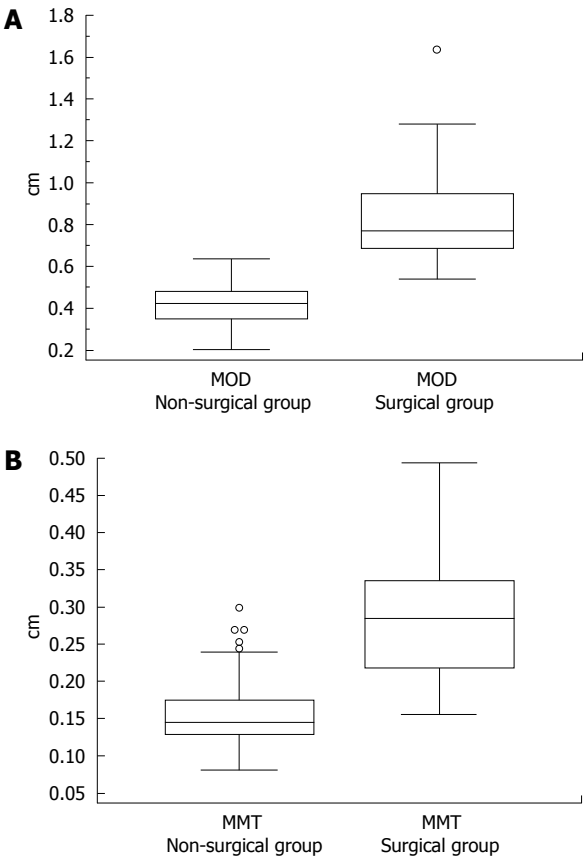

Figure 2 Data comparison graphs of MOD (A) and MMT (B) in box-and-whisker plots. The median MOD and the median MMT were significantly different ($P < 0.0001$) between two groups.

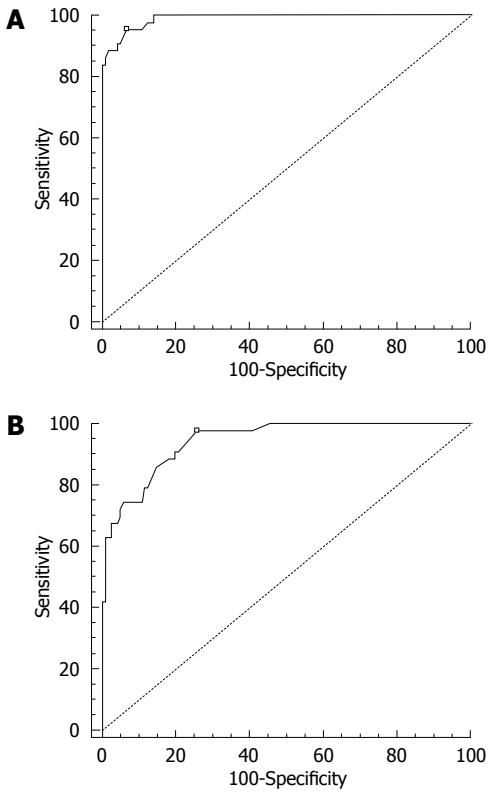

Figure 3 ROC curves of MOD (A) and MMT (B). The optimal cut-off points are marked as white square boxes on the graphs.

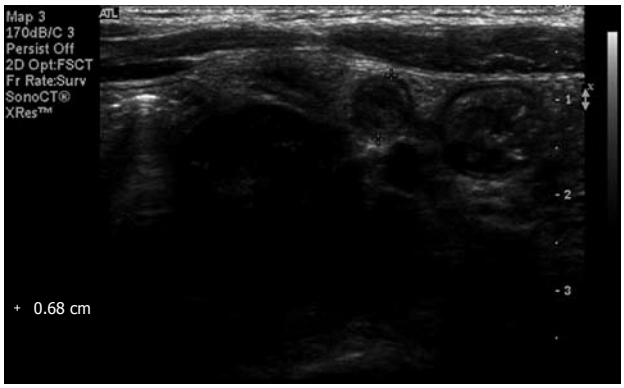

Figure 4 Cross-sectional image of a distended normal appendix in a 7-year-old boy. The MOD of the appendix was 0.65 cm. The intra-luminal hyperechogenicity was due to gas and fecal materials. We diagnosed this as normal appendix, and the symptoms spontaneously resolved.

and 0.10-0.58 cm in the surgical appendix group. The median MMT of 0.15 cm (95% CI: 0.13-0.17 cm) in the non-surgical appendix group was significantly different ($P < 0.0001$) from the median MMT of 0.33 cm (95% CI: 0.30-0.38 cm) in the surgical appendix group. The data comparison graphs between the two groups were presented in Figure 2B.

By ROC curve analysis, the optimal cut-off MOD was 0.57 cm with 89.6% sensitivity, 93.2% specificity and a 13.1 positive likelihood ratio (Figure 3A). The optimal cut-off MMT was 0.22 cm with 84.6% sensitivity, 95.8% specificity and a 20.1 positive likelihood ratio (Figure 3B).

DISCUSSION

According to previous reports[1-4,11], the diagnosis of acute appendicitis by US is based on the following findings; the MOD of the appendix > 0.6 cm; the appendix cannot be compressed with manual pressure by the examiner; the cross-sectional shape of the appendix is round rather than oval; there is an absence of gas in the appendiceal lumen; and there is hyperperfusion of the appendiceal wall on a Doppler study. However, the most credible criterion, the MOD, may be exaggerated and inaccurate in certain conditions (Figure 4)[5-7].

Earlier, Park *et al*[10] suggested that the MMT may have a role as a useful adjunctive measurement, especially for patients with fecal-impacted, non-inflammatory appendixes. As well as this study, there have been several research studies measuring the MMT in children. Simonovský[8] reported that the difference in the normal appendiceal MMT between a group of young children and adolescents and an adult group was marginally significant ($P = 0.042$). In addition, the investigator stated that an MMT < 3 mm should be regarded as normal in children aged six years or younger. Wiersma *et al*[9] also reported the sizes of the MOD and the MMT of a normal appendix in children as 0.21-0.64 cm and 0.11-0.27 cm, respectively. However, they studied only normal appendixes.

In our study, we examined data for both diseased and disease-free appendixes on a large scale, and we compared the MOD and MMT between the surgical appendix and the non-surgical appendix. In a statistical

analysis, both the MMT and the MOD had diagnostic value for acute appendicitis in children.

In addition, we were able to obtain the optimal cut-off MOD and MMT values for diagnosing acute appendicitis in pediatric patients. By ROC curve analysis, the optimal cut-off MOD was 0.57 cm with 89.6% sensitivity, 93.2% specificity and a 13.1 positive likelihood ratio. The optimal cut-off MMT was 0.22 cm with 84.6% sensitivity, 95.8% specificity and a 20.1 positive likelihood ratio. Considering that literature during the last three years has reported that the sensitivity and the specificity for diagnosis of acute appendicitis with US were 80%-100% and 86.5%-100%[10,12-19], our study showed acceptable sensitivity and specificity. While we expected that the MMT would be more sensitive than the MOD, the MMT was less sensitive but more specific than the MOD. We presumed that the MMT could only decrease the false positive ratio of the MOD and could not affect the false negative ratio as the MOD was more sensitive. Therefore, in children, an MOD > 0.57 cm suggests the presence of acute appendicitis and an MMT > 0.22 cm enhances the possibility of having acute appendicitis.

There were several limitations to this study. At first, because our data were obtained only from visible appendixes on US, cases of perforated appendicitis were excluded. Secondly, we categorized patients according to the results of surgery, and therefore cases of chronic or abortive appendicitis may be categorized in the non-surgical appendix group. Thirdly, because the examiner was not one radiologist, inter-observer variance still exists.

In conclusion, the MOD and the MMT are reliable criteria for diagnosis of acute appendicitis in children. An MOD > 0.57 cm and an MMT > 0.22 cm are the optimal criteria.

COMMENTS

Background

US became a reliable modality for diagnosing acute appendicitis, especially in doubtful cases for clinicians. When the maximal outer diameter (MOD) of the appendix is larger than 0.6 cm, radiologists suggest the presence of acute appendicitis. However, because the MOD may be larger than 0.6 cm without acute inflammation, the maximal mural thickness (MMT) of the appendix has been evaluated as another size criterion.

Research frontiers

Dr. Wiersma and colleagues in Juliana Children's hospital in The Netherlands reported the sizes of the MOD and the MMT in the normal appendix. Dr. Park and colleagues in Kwandong University Myongji Hospital in South Korea suggested that the MMT may play a role as a useful measurement for the fecal-impacted, non-inflammatory appendix. However, they included only normal appendixes. The authors examined data from both diseased and disease-free appendixes on a large scale to evaluate the MMT and the MOD and to determine the optimal cut-off values of these measurements in the diagnosis of acute appendicitis in children.

Applications

The MOD and the MMT are reliable criteria for diagnosing acute appendicitis in children. An MOD > 0.57 cm and an MMT > 0.22 cm are the optimal criteria.

Peer review

The authors demonstrated the usefulness of measurements of MOD and MMT in diagnosing pediatric acute appendicitis. They compared the MOD and MMT between surgical and non-surgical appendix groups and showed significant differences between the two groups. They also determined optimal cut-off value of MOD and MMT for diagnosis of acute appendicitis. The manuscript was well organized and well written.

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BRIEF ARTICLES

Experience of limited pancreatic head resection for management of branch duct intraductal papillary mucinous neoplasm in a single center

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Abstract

AIM: To share our surgical experience and the outcome of limited pancreatic head resection for the management of branch duct intraductal papillary mucinous neoplasm (IPMN).

METHODS: Between May 2005 and February 2008, nine limited pancreatic head resections (LPHR) were performed for IPMN of the pancreatic head. We reviewed the nine patients, retrospectively.

RESULTS: Tumor was located in the uncinate process of the pancreas in all nine patients. Three patients had stents inserted in the main pancreatic duct due to injury. The mean size of tumor was 28.4 mm. Postoperative complications were found in five patients: 3 pancreatic leakages, a pancreatitis, and a duodenal stricture. Pancreatic leakages were improved by external drainage. No perioperative mortality was observed and all patients are recorded alive during the mean follow-up period of 17.2 mo.

CONCLUSION: In selected patients after careful evaluation, LPHR can be used for the treatment of branch duct type IPMN. In order to avoid pancreatic ductal injury, pre- and intra-operative definite localization and careful operative techniques are required.

Key words: Limited pancreatic head resection; Branch duct type; Intraductal papillary mucinous neoplasm

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INTRODUCTION

Branch duct type intraductal papillary mucinous neoplasm (IPMN) has a low malignant potential and is more frequently located in the head of the pancreas[1-4]. When this lesion is located in the pancreatic head, the conventional treatment for IPMN has been pancreaticoduodenectomy (PD). Also, partial pancreatic resection is feasible as a treatment for small branch duct type IPMN, which shows less aggressive behavior.

In recent years, some surgeons have advocated limited pancreatectomy for management of benign IPMN and regarding this the following procedures have been reported in several papers: inferior head resection of the pancreas[5], partial pancreatic head resection[6], uncinate process resection[7,8], single branch resection of the pancreas[9], and ductal branch oriented minimal pancreatectomy[10]. It had been expected that there would be advantages of minimal pancreatic parenchymal loss and prevention of functional insufficiency by performing limited pancreatectomy. However, recommendations and reports of postoperative complications and clinical outcomes following these procedures have been limited. Therefore we report a single center surgical experience and short-term outcome of limited pancreatic head resection (LPHR) for the management of branch duct type IPMN.

MATERIALS AND METHODS

From May 2005 to February 2008, a retrospective review

Table 1 Clinical aspect of patients undergoing LPHR

Case No.	F/U (mo)	Complication	Pathology	Size (mm)	Preop Dx	Pancreatogram	Age	Gender
1	24	P-leakage	Adenoma	30	MCN	ERCP	69	F
2	20	Pancreatitis	Adenoma	25	Cystic.n	ERCP	67	M
3	22	P-leakage	Adenoma	20	IPMN	MRCP	71	M
4	22	D-stricture	Adenoma	22	Cystic.n	ERCP	72	M
5	19	-	Adenoma	25	MCN	ERCP	50	F
6	14	-	Adenoma	42	IPMN	ERCP	69	M
7	12	-	Adenoma	27	IPMN	MRCP	60	M
8	12	P-leakage	Adenoma	30	IPMN	MRCP	42	M
9	10	-	Adenoma	35	IPMN	MRCP	67	F

ERCP: Endoscopic retrograde cholangiopancreatogram; MRCP: Magnetic resonance cholangiopancreatogram; MCN: Mucinous cystic neoplasm; Cystic.n: Cystic neoplasm of pancreas; IPMN: Intraductal papillary mucinous neoplasm; P-leakage: Pancreatic leakage; D-stricture: Duodenal stricture.

was undertaken of 12 patients who underwent partial pancreatectomy for IPMN at our institution, among whom, nine patients underwent LPHR. In-hospital clinical course and method of operation, postoperative complications and follow-up data were analyzed.

Assessment of the tumor location was carried out before surgery using computed tomography (CT) in all nine cases, upon suspicion of IPMN.

Additional evaluations were routinely performed with endoscopic retrograde cholagiopancreatography (ERCP) or magnetic resonance cholagiopancreatography (MRCP) for evaluating the pancreatic duct, which were confirmed during surgery through direct visualization of lesion by dissection of pancreas head and intraoperative ultrasonography (IOUSG). All pancreatectomies were performed by a single surgeon. Pancreatic parenchymal control was performed using bipolar coagulator for fine dissection and easy bleeding control. All nine resected tumors were examined by a single pathologist with regard to resection margin and tumor characteristics during operation. The definition of postoperative pancreatic leakage was in accordance with the International Study Group on Pancreatic Fistula (ISGPF) definition[11].

Indication for limited pancreatectomy

Prior to 2005, PD was the standard treatment for IPMN of the pancreas head. Sugiyama *et al*[12] reported that size > 30 mm and presence of mural nodules were the strongest predictors of malignancy in branch duct IPMN. But our previous study revealed that many of < 30 mm resected branch duct IPMN were malignant[13]. For these reason, under 30 mm-sized branch duct IPMN were resected at our center, and < 20 mm lesions were observed with close follow-up. Since 2005, LPHR procedure was tried for those lesions > 20 mm, mainly located in the uncinate process in which the main pancreatic duct was intact on imaging study, and branched IPMN was suspected on pancreatogram.

Operative procedure

Pancreatic head was exposed by omentectomy and superior mesenteric vein (SMV) branch ligation such as in PD. After the exposure, exploration of the lesion and main pancreatic duct was carried out using IOUSG. Preoperative or intraoperative pancreatic stents were not used before resection. Bipolar coagulator was used for fine dissection of pancreas head.

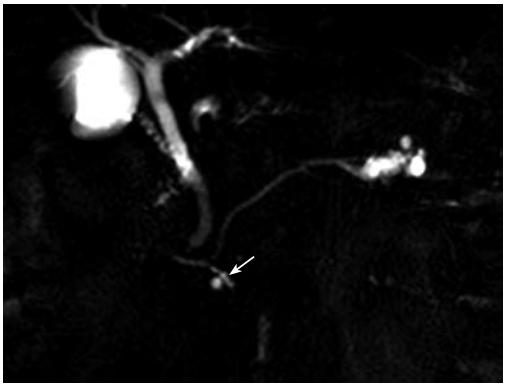

Figure 1 MRCP showing connection between main pancreatic duct and cystic lesion in uncinate process (arrow).

RESULTS

Clinical findings

The mean age of the patients was 63 years (range: 42-72 years), with six male patients. Six patients presented clinical symptoms and incidental lesions were found in three patients.

All nine patients underwent multidetector abdominal CT, four of nine patients underwent MRCP, and another five patients underwent ERCP. All nine tumors were located in the uncinate process, and two patients had another lesion in the tail portion of the pancreas. Five patients were confirmed IPMN by imaging studies, another four patients were suspected IPMN or other cystic neoplasm including mucinous cystic neoplasm (MCN). Clinical profiles are summarized in Table 1.

MRCP finding

Three quarters of the cases of MRCP showed connection between the main pancreatic duct and the cystic lesion in the uncinate process (Figure 1). These findings aroused suspicion of branched type of IPMN, and LPHR was planned.

LPHR

Uncinate process resection or ductal branch-oriented

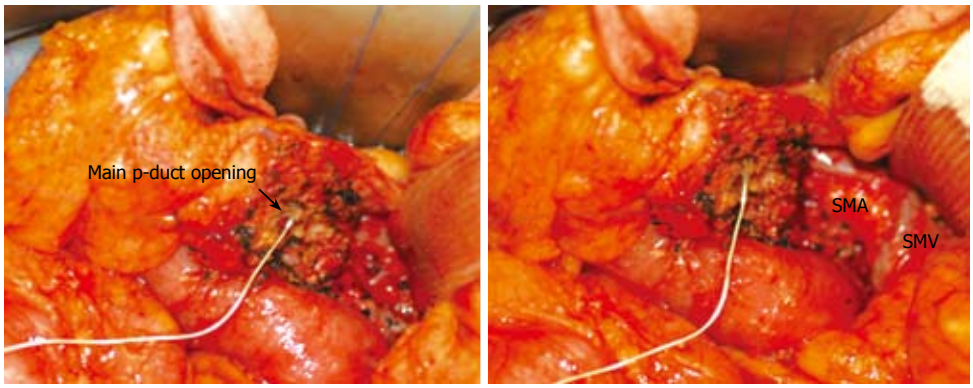

Figure 2 Main pancreatic duct was torn during uncinate process resection (arrow).

minimal pancreatectomy[7,10] were performed in six patients. Single-branch resection[9] was performed in three patients. Two of nine patients underwent additional distal pancreatectomy for pancreatic tail IPMN. The Wirsung duct was damaged in three of the six patients who underwent uncinate process resection or ductal branch-oriented minimal pancreatectomy in which internal silastic stents were inserted and primary repair was carried out (Figure 2). After pancreatectomy, we confirmed the pathologic diagnosis with resection margin.

Pathology

The mean tumor size was 28.4 mm (range 20-42 mm). All nine tumors were confirmed as intraductal papillary mucinous adenoma and resection margins were free of tumor.

Postoperative course

The mean number of hospital days was 21.1 d (range: 8-48 d). There was no mortality and five morbidities. Pancreatic leakage occurred in three patients, two of which involved injured pancreatic duct during operation and an inserted silastic internal pancreatic stent. The third ductal injury was not detected during operation. Pancreatic leakage was detected on postoperative days 4 and 5 respectively. One patient was discharged on postoperative day 32 with a Jackson-Pratt (JP) drain which was removed on day 62 after drain amylase was normalized. Another patient with re-exploration at diagnosis of leakage showed severe inflammation, because of which an external drain (sump drain) was added. Both patients were discharged at postoperative day 43 after removal of the drain. In the case of non-injury of the pancreatic duct, drain amylase increased after operation but normalized at postoperative day 15. One pancreatitis and one duodenal stricture were observed. Duodenal stricture was improved after gastrojejunostomy. The mean follow-up time was 17.2 mo during which there were no recurrences or metastases.

DISCUSSION

Despite the small number of cases included in this study, to our knowledge this study and evaluation of the nine cases of LPHR for branched IPMN is so far the largest amongst related studies. It can be said that partial pancreatic head resection can be a better treatment option than conventional PD for branch duct IPMN on pancreas head under free resection margin. PD may present as surgical overkill for benign and low-grade malignant tumors such as branch duct type IPMN of the pancreatic head. Such procedures result in a significant loss of normal pancreatic parenchyma with subsequent impairment of exocrine and endocrine pancreatic functions[14-16]. Resection of the distal bile duct in patients undergoing PD requires a bilio-enteric anastomosis, which increases the risk of ascending cholangitis and subsequent intrahepatic abscesses[17]. Following PD, the incidence of diabetes mellitus varies between 15% and 40%[18,19]. It can be noted in reports of recent papers that there is unchanged endocrine and exocrine pancreatic function following segmental pancreatic resection[20-22].

Ventral pancreatectomy was performed for management of cystic tumor as limited pancreatic head resection in 1993[23]. This procedure resected too great a width of normal pancreas for a small lesion and reconstructed pancreatic duct and bile duct by entric-anastomosis. Thereafter, pancreatic duct preserving procedures were reported, such as inferior pancreatic head resection, and uncinate process resection[5,7,8]. More minimal pancreatic head resections were performed such as single branch resection of the pancreas, and ductal branch-oriented minimal pancreatectomy[9,10].

Pancreatic leakage is one of the most frustrating complications after limited pancreatic resection. Although this study showed no mortality, three cases of pancreatic leakages occurred. Three pancreatic injuries were found during pancreatectomy. Firstly, the main pancreatic duct was injured after pancreatic dissection from the superior mesenteric vein (SMV) where mucin leaked from a small ductal opening in which a stent was inserted. Secondly, due to ductal tearing, the main pancreatic duct opening was widened during which a stent was inserted through the opening site. Lastly, minutely injured duct was repaired without stent insertion, without leakage after operation. Sata *et al*[9] experienced pancreatic leakages which were managed by the insertion of an endoscopic naso-pancreatic drainage tube. In segmental pancreas resection, pancreatic leakage rates reach up to 40%[20-22].

Generally, benign branch duct type IPMN in the pancreas head, especially the uncinate process, does not involve the main pancreatic duct. However IPMN too close to the main duct, or a large mass, need to be carefully evaluated. For safe dissection during operation without injuring the main pancreatic duct, pre- or intra-

operative pancreatic duct evaluation is crucial. IOUSG or MRCP were insufficient for detecting the pancreatic duct or distance of duct to mass during operation. Although all nine patients had IOUSG performed, three pancreatic injuries occurred. Takada *et al*[7] applied preoperative pancreatic duct stents guided by ERCP, during which the patients did not experience pancreatic leakage. They mentioned that the purpose of the stent was intraoperative identification of pancreatic duct with protection against iatrogenic injury, and postoperative drainage for minimizing pancreatic fistula. Yamaguchi *et al*[10] proposed the placement of a main duct tube for preventing transient stenosis of pancreatic duct. However in this study, preoperative stents were not applied. We speculated that the pancreatic drainage tube produced a pancreatitis.

LPHR does not necessitate anastomosis between the pancreatic duct, bile duct and the bowel. A disadvantage of LPHR was the higher rate of leakage (33.3%). In order to avoid pancreatic ductal injury, preoperative or intraoperative definite localization and careful surgical techniques were important. If the pancreatic duct was injured during operation, internal drainage procedure was necessary. If the disadvantage of ductal injury can be overcome, LPHR can be a useful procedure for the treatment of branch duct type IPMN in selected patients.

COMMENTS

Background

Branch duct type intraductal papillary mucinous neoplasm (IPMN) has a low malignant potential. Limited pancreatectomy is advocated for those lesions, thereby reducing the risk of functional insufficiency and morbidity in extensive resection.

Innovations and breakthroughs

Recommendations and reports of clinical outcomes following limited pancreatic head resection procedures were limited. Several case reports have been published. Despite the small number of cases included in this study, evaluation of the nine cases of limited pancreatic head resections (LPHR) for branched IPMN is so far the largest amongst related studies.

Applications

Disadvantage of LPHR was the higher rate of leakage. If the disadvantage of ductal injury can be overcome, LPHR can be a useful procedure for the treatment of branch duct type IPMN in selected patients.

Peer review

This interesting paper explores the possibility of limited resections for branch duct-type IPMN. But, three of 9 patients also developed a pancreatic fistula; a rate that is also higher than after pancreaticoduodenectomy.

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BRIEF ARTICLES

Ampullary carcinoma: Effect of preoperative biliary drainage on surgical outcome

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Abstract

AIM: To evaluate the influence of preoperative biliary drainage on morbidity and mortality after surgical resection for ampullary carcinoma.

METHODS: We analyzed retrospectively data for 82 patients who underwent potentially curative surgery for ampullary carcinoma between September 1993 and July 2007 at the Singapore General Hospital, a tertiary referral hospital. Diagnosis of ampullary carcinoma was confirmed histologically. Thirty-five patients underwent preoperative biliary drainage (PBD group), and 47 were not drained (non-PBD group). The mode of biliary drainage was endoscopic retrograde cholangiopancreatography (n = 33) or percutaneous biliary drainage (n = 2). The following parameters were analyzed: wound infection, intra-abdominal abscess, intra-abdominal or gastrointestinal bleeding, septicemia, biliary or pancreatic leakage, pancreatitis, gastroparesis, and re-operation rate. Mortality was assessed at 30 d (hospital mortality) and also long-term. The statistical endpoint of this study was patient survival after surgery.

RESULTS: The groups were well matched for demographic criteria, clinical presentation and operative characteristics, except for lower hemoglobin in the non-PBD group (10.9 ± 1.6 *vs* 11.8 ± 1.6 in the PBD group). Of the parameters assessing postoperative morbidity, incidence of wound infection was significantly less in the PBD than the non-PBD group [1 (2.9%) *vs* 12 (25.5%)]. However, the rest of the parameters did not differ significantly between the groups, i.e. sepsis [10 (28.6%) *vs* 14 (29.8%)], intra-abdominal bleeding [1 (2.9%) *vs* 5 (10.6%)], intra-abdominal abscess [1 (2.9%) *vs* 8 (17%)], gastrointestinal bleeding [3 (8.6%) *vs* 5 (10.6%)], pancreatic leakage [2 (5.7%) *vs* 3 (6.4%)], biliary leakage [2 (5.7%) *vs* 3 (6.4%)], pancreatitis [2 (5.7%) *vs* 2 (4.3%)], gastroparesis [6 (17.1%) *vs* 10 (21.3%)], need for blood transfusion [10 (28.6%) *vs* 17 (36.2%)] and re-operation rate [1 (2.9%) *vs* 5 (10.6%)]. There was no early mortality in either group. Median survival was 44 mo (95% CI: 34.2-53.8) in the PBD group and 41 mo (95% CI: 27.7-54.3; P = 0.86) in the non-PBD group.

CONCLUSION: Biliary drainage before surgery for ampullary cancer significantly reduced postoperative wound infection. Overall mortality was not influenced by preoperative drainage.

Key words: Ampullary carcinoma; Preoperative biliary drainage; Postoperative complications

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INTRODUCTION

In patients with ampullary cancer who undergo surgical resection, obstructive jaundice is associated with a higher risk of postoperative complications than in non-jaundiced patients[1]. The impact of jaundice on postoperative morbidity and mortality is well known. However the routine use of preoperative biliary

drainage (PBD) remains controversial. The potential advantages of preoperative stenting include improved nutritional, metabolic and immune function and the possibility of reduced postoperative morbidity and mortality[1]. Opponents of PBD argue that it increases infective complications and morbidity[2,3]. However, there are certain clinical situations such as acute suppurative cholangitis and severe malnutrition in which urgent biliary drainage is indicated and can be life-saving[4]. It is not clear whether the procedure itself or its complications influence the morbidity after surgical resection. The optimal duration of preoperative drainage also remains unknown. Although several reports have been published, there are still no clear guidelines regarding the use of PBD in these patients. We analyzed our patients to assess the influence of PBD on postoperative outcome following pancreaticoduodenectomy (PD) for ampullary tumors.

MATERIALS AND METHODS

We reviewed retrospectively the records of all patients who underwent definitive surgery for carcinoma involving the ampulla of Vater between and September 1993 and July 2007 at the Singapore General Hospital, a tertiary care referral and teaching hospital.

Ampullary cancer was defined as a tumor arising from the ampulla, with evidence of invasion and histopathological signs of neoplasia. In the case of large tumors with involvement of adjacent duodenum or pancreas, the tumor was classified as ampullary if it was centered on the ampulla. Cancers of pancreatic, duodenal and choledochal origin were excluded.

Details of patients who underwent PD (Whipple's operation) or pylorus-preserving pancreaticoduodenectomy (PPPD) for ampullary tumors were entered into a database that included patient characteristics, details of biliary stenting, procedure-related infective complications, surgery, morbidity and mortality. The operations were performed in the Department of Surgery, Singapore General Hospital.

Morbidity

Pancreatic leakage was diagnosed when > 50 mL of drainage fluid, with a serum amylase concentration > 3 times the upper limit of normal was obtained on or after postoperative day 5, or when pancreatic anastomotic disruption was demonstrated radiologically[3]. Wound infection was defined as spontaneous or surgically released purulent discharge that was positive for bacterial growth on culture. Bile leakage was defined as a bilirubin concentration in the drainage fluid that exceeded that in the serum, which resulted in a change of clinical management or the occurrence of a bilioma that required drainage. Infectious morbidity was defined as any complication with evidence of associated localized or systemic infection indicated by fever, leukocytosis and positive culture.

Intra-abdominal or gastrointestinal bleeding was defined as bleeding with either hemodynamic instability or patients who required > 2 U blood transfusion or who required re-operation. Intra-abdominal abscess was defined as purulent discharge with positive cultures from abdominal drains placed at surgery, or as fluid collection that required a drainage procedure. Delayed gastric emptying was defined as inability to tolerate a regular diet for more than seven postoperative days, the need for nasogastric tube drainage for seven or more days postoperatively, or the need for tube reinsertion after removal.

Mortality

Hospital deaths were defined as those that occurred within 30 d of operation or as a direct result of postoperative complications. Late mortality was defined as mortality after 30 d during the follow-up period.

Pathological data

Pathological data were obtained from the patients' medical records and the surgical pathology files. Histological grade, type and the presence of malignant change were noted.

Statistical analysis

Results were expressed as medians and ranges or as numbers and percentages of patients. Two-tailed *t* test and χ^2 test were used for data analysis. SPSS version 13.0 statistical software was used (Chicago, IL, USA) for analysis. Differences were considered statistically significant at $P < 0.05$. The major statistical endpoint of this study was patient survival. Event time distributions for this endpoint were estimated using the method of Kaplan and Meier[5] and compared using the log-rank statistic.

RESULTS

Eighty-two patients were included in the study. Seventy-nine had Whipple's operation and three had PPPD for ampullary tumors. Forty patients (48.8%) were male and 42 (51.2%) were female. Thirty-five (42.7%) patients underwent preoperative biliary plastic stent insertion. There were no inherent differences between the two groups with regard to the decision to proceed with PBD stent insertion. Demographics of the 82 patients with ampullary cancer are summarized in Table 1. The two groups were comparable with regard to sex, age, stage and grading of the ampullary cancer, and diabetes mellitus. Patients who underwent PBD had a significantly higher level of hemoglobin (11.8 ± 1.5 g/dL) on preoperative laboratory evaluation compared with non-stented patients (10.9 ± 1.6 g/dL) ($P < 0.05$). The majority of biliary stents were placed endoscopically, with two patients having percutaneous transhepatic biliary drainage. Numbers were too small to undergo statistical analysis between the types of biliary decompression; thus, "stents" included all patients who underwent PBD. The time interval between biliary drainage and surgery was 39 d (range: 10-89 d). There was a decrease in bilirubin in all patients, with the mean reduction being 47.6 μmol/L. All patients were given intravenous antibiotics at the time of surgery.

Table 1 Patient variables in the PBD and non-PBD groups

	PBD	Non-PBD	Significance
Patients	35	47	
Median age (yr)	65 (23-84)	62 (38-84)	0.91
Sex ratio (M/F)	14/21	26/21	0.28
Mean serum bilirubin	112.4 ± 116.1	91.6 ± 110.2	0.39
Mean hemoglobin	11.8 ± 1.5	10.9 ± 1.6	0.03
Diabetes mellitus	7	12	0.66
Mean albumin	30.5 ± 5.7	31.1 ± 6.2	0.77

Table 2 Morbidity and mortality in the two groups

Morbidity	PBD	Non-PBD	Significance
Sepsis	10	14	0.91
Wound infection	1	12	0.01
Intra-abdominal bleeding	1	5	0.36
Intra-abdominal abscess	1	8	0.09
Gastrointestinal bleeding	3	5	0.95
Bile leakage	2	3	0.73
Pancreatic leakage	2	2	0.73
Delayed gastric emptying	6	10	0.85
Reoperation	1	5	0.36
Hospital death	0	0	0.00
Late mortality	10	13	0.15

All postoperative complications and subpopulation analyses between PBD and non-PBD groups are shown in Table 2. Postoperative sepsis occurred in 29.3% (24/82) of the patients. Six patients had postoperative bleeding that required re-exploration. There were 13 (15.7%) patients with wound infection (one in the PBD and 12 in the non-PBD group), and analysis demonstrated a significantly higher occurrence of wound infection in the non-PBD group ($P = 0.01$).

Tumor grading and histology

Of the 82 infiltrating carcinomas, 21 (25.6%) were well-differentiated, 49 (59.8%) were moderately differentiated, and 12 (14.6%) were poorly differentiated. The most common histological type was intestinal (52/82, 63.4%), followed by pancreaticobiliary (22/82, 26.8%) and colloid (3/82, 3.7%). Five carcinomas had a histological type that was classified as "other".

Mortality and survival

There were no hospital deaths in either group. Twenty-three patients died (28%) during the study period. Median survival was 44 mo (95% CI: 34.2-53.8) in the PBD group and 41 mo (95% CI: 27.7-54.3; $P = 0.86$) in the non-PBD group. There was no significant difference in terms of survival between the groups (Figure 1).

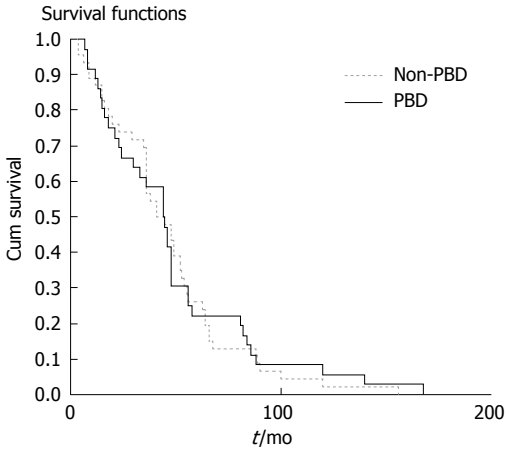

Figure 1 Survival functions for PBD and non-PBD groups.

DISCUSSION

Ampullary tumors share a similar clinical presentation in which jaundice is the predominant symptom. The main initial objectives in these patients are to obtain a precise diagnosis and resolution of jaundice. In an attempt to reach these goals, many treatment modalities have been proposed and applied in recent years. All have been oriented to the necessity of reducing jaundice preoperatively and to preventing perioperative complications, in most cases caused by cholestasis. We found an increase in the rate of wound infection in the perioperative period in the non-PBD group. Malnourishment and malignant obstructive jaundice predispose a patient to wound dehiscence by slowing healing, and increasing the rate of wound infection. A study by Irvin *et al*[6] has suggested that malignant disease may be an important factor in the pathogenesis of wound complications in patients with jaundice. Wound dehiscence or incisional hernia occurred in 59.1% of patients with obstructive jaundice that resulted from malignant disease, but patients with jaundice caused by biliary stones or benign pathology did not develop these complications.

Other factors have been confirmed as having a significant effect on the development of postoperative wound infection, in terms of patient characteristics including diabetes and anemia[7]. Patients with diabetes are more susceptible to wound infection because of impaired neutrophil chemotaxis and phagocytosis. In our series, 25% of non-PBD patients had diabetes compared to 20% in the PBD group, which, although not statistically significant, may have influenced the outcome of surgery. Furthermore the non-PBD group had a hemoglobin level that was significantly lower than that in the PBD group. Impaired wound healing in patients with obstructive jaundice has also been postulated to be caused by an altered immune response, with elevated tumor necrosis factor α activity secondary to circulating endotoxemia[8].

Grande *et al*[9] have compared wound healing in the presence and absence of obstructive jaundice. They used prolyl hydroxylase activity as a marker for collagen synthesis, and found it to be significantly elevated in patients who underwent biliary drainage for benign or malignant biliary obstruction. These patients had better wound healing.

Three common methods of PBD are percutaneous, endoscopic and surgical instrumentation. The endoscopic insertion of a stent through the papilla is a method of draining an obstructed biliary system during endoscopic retrograde cholangiopancreatography (ERCP). In animal studies comparing internal and external biliary drainage, animals undergoing internal drainage experienced

increased survival[10,11], decreased sepsis[12], renal failure[11], and more rapid recovery of immune function[13,14], compared with those undergoing external drainage. Endoscopic biliary drainage is now considered an effective, if not the preferable, treatment for the palliation of malignant biliary tract obstruction[15,16].

To the best of our knowledge, this is the first study to analyze ampullary cancer in the context of PBD. Previous studies have looked at peri-ampullary cancer. Trede and Schwall's[17] retrospective analysis of 150 patients with jaundice undergoing partial or total pancreatectomy revealed a complication rate of 31% and four deaths in 68 patients with no drainage, compared with 17% complications and one death in 82 patients with PBD. Evidence in support of preoperative biliary drainage comes from a prospective randomized trial by Lygidakis *et al*[18], which assigned 38 patients to either PBD (15 ± 2 d) by endoscopic internal stenting, or no PBD. The authors reported a 16% complication rate and no deaths in 19 patients undergoing PBD, compared with 70% complications and two deaths in 19 patients without drainage. Although this difference was highly significant, the investigators scored positive intraoperative blood and bile cultures as a complication. The clinical relevance of such findings is not clear. Smith *et al*[19] have identified prospectively 155 patients who underwent partial PD and found no survival difference between PBD and non-PBD groups. However, the authors concluded that the presence of jaundice at the time of resection had an adverse impact on early postoperative survival. Therefore, preoperative resolution of jaundice following biliary stenting predicted more favorable early survival outcome.

In contrast, a prospective randomized study by Lai *et al*[20] revealed no significant benefit in patients undergoing PBD, and included a wide variety of pathologies, but details of specific complications were not delineated clearly. Choi *et al*[21] have shown that PBD compromised hepatic excretory function, as represented by a slow rate of decrease in serum bilirubin. Limongelli *et al*[22] have revealed that PBD predisposes to a positive intraoperative bile culture, which increases the risk of developing infectious complications and wound infection after pancreatic surgery. Povoski *et al*[23] have reviewed retrospectively 240 consecutive cases of PD. Postoperative morbidity and mortality rates were higher in the PBD group, and they have suggested that PBD should be avoided whenever possible in patients with potentially resectable pancreatic and peripancreatic lesions. Therefore, based on data that, at best, provide mixed results, why should the patients still undergo PBD? First, most patients with ampullary cancer at the time of diagnosis have symptomatic jaundice with pruritus, and some degree of abdominal pain. To some extent, it is hoped that stenting will provide symptomatic improvement. Second, patients who present with acute cholangitis require prior PBD before undergoing definitive surgery.

In contrast, endoscopic biliary drainage before surgery is not a widely accepted procedure among pancreatic surgeons. Potential disadvantages of PBD include those inherent to ERCP such as pancreatitis, bleeding, cholangitis and duodenal perforation. In addition, endoscopic biliary stenting has been shown to generate a severe inflammatory reaction in the bile duct[24], which may make surgical resection more difficult. This was not substantiated by our study, with its comparable mortality and significantly reduced wound infection in the PBD group.

In conclusion, our experience confirms that PBD in patients with obstructive jaundice due to ampullary cancer results in a reduction in wound infection following surgical resection compared with patients not undergoing PBD. Morbidity other than wound infection and mortality were similar with or without PBD. We believe that a detailed prospective randomized trial including a cost analysis in a well-defined and well-matched group of patients undergoing operation for ampullary cancer is warranted, to further evaluate the effectiveness of routine preoperative PBD.

COMMENTS

Background

The effectiveness of preoperative biliary drainage (PBD) in pancreaticoduodenectomy is still extensively debated because of the various conflicting postoperative outcomes, which include benign or malignant, pancreatic or peripancreatic, and ampullary or periampullary lesions.

Research frontiers

The impact of jaundice on postoperative complications is well recognized. However, whether PBD with improvement of jaundice affects surgical outcomes remains controversial.

Innovations and breakthroughs

This is believed to be the first study to investigate the impact of PBD in resectable ampullary cancer patients. Other studies have shown mixed results with regard to postoperative morbidity and mortality amongst ampullary and periampullary cancer.

Applications

The authors conducted the study amongst 82 patients with ampullary cancer and 35 of them had PBD, with a significant reduction in postoperative wound infection rate. A further randomized prospective control study should be conducted in resectable ampullary cancer patients undergoing PBD to look into this positive outcome.

Peer review

This paper addresses an important clinical issue: whether preoperative biliary drainage influences the outcome of resectional surgery for ampullary carcinoma. The results are presented reasonably clearly. The discussion compares the outcomes of this study with other relevant studies and explores the possible reasons for the findings.

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BRIEF ARTICLES

Microproteinuria for detecting calcineurin inhibitor-related nephrotoxicity after liver transplantation

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Abstract

AIM: To investigate whether microproteinuria could be used as an early and sensitive indicator to detect calcineurin inhibitor (CNI)-related nephrotoxicity after liver transplantation.

METHODS: All liver transplant recipients with normal serum creatinine (SCr) and detectable microproteinuria at baseline were included in this study. The renal function was monitored by the blood clearance of ^{99m}Tc-diethylenetriaminepentaacetic acid every 6 mo. Microproteinuria, SCr and blood urea nitrogen (BUN) were measured at entry and at subsequent follow-up visits. The patients were divided into different groups according to the mean values of glomerular filtration rate (GFR) at the follow-up time points: Group 1, GFR decreased from baseline by 0%-10%; Group 2, GFR decreased from baseline by 11%-20%; Group 3, GFR decreased from baseline by 21%-40%; Group 4, GFR decreased from baseline by > 40% and/or SCr was increasing.

RESULTS: A total of 143 patients were enrolled into this study (23 females and 120 males). The mean follow-up was 32 mo (range 16-36 mo). Downward trends in renal function over time were observed in the study groups. SCr and BUN increased significantly only in Group 4 patients ($P < 0.001$). β2-microglobulin (β2m) and α1-microglobulin (α1m) significantly increased with the subtle change of renal function in recipients who were exposed to CNI-based immunosuppression regimens. The reductions in GFR were closely correlated with elevated α1m ($r^2 = -0.728$, $P < 0.001$) and β2m ($r^2 = -0.787$, $P < 0.001$).

CONCLUSION: β2m and α1m could be useful as early and sensitive indicators of CNI-induced nephrotoxicity.

Key words: Microproteinuria; Liver transplantation; Calcineurin inhibitors; Nephrotoxicity

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INTRODUCTION

Calcineurin inhibitors (CNIs) have improved survival significantly after liver transplantation, but nephrotoxicity is an adverse effects common to both cyclosporine and tacrolimus[1-3]. Deterioration of renal function with CNI therapy has been widely reported in liver transplant

recipients[4-6]. A recent analysis of 36 849 liver transplant recipients performed in the United States between 1990 and 2000 revealed an 18.1% incidence of chronic renal failure after 5 years, with CNI dose and duration of exposure correlated with subsequent renal damage[7].

The monitoring of transplant patients, however, is still dependent on somewhat old methodologies: serum creatinine (SCr) levels, total urine output, blood urea nitrogen (BUN), or calculated glomerular filtration rate (GFR). However, these tests are particularly problematic as they do not have sufficient specificity, sensitivity, or accuracy to allow appropriate and timely interventions[8]. Additionally, CNIs are characterized by a narrow therapeutic index, so renal damage is often irreversible when these indices become abnormal, or the indices can still fluctuate in the normal range while renal insufficiency is obvious. Given these limitations, more and more transplant specialists are looking to emerging fields, such as proteomics and metabolism, to improve the current situation. Microproteinuria, as a hallmark of reflecting early changes in the glomeruli and proximal tubular function, can be used as an accurate predictor to monitor early changes in renal function[9,10].

The aim of this prospective study was to find out whether microproteinuria could be used as an early and sensitive indicator to monitor CNI-related nephrotoxicity in liver transplant recipients. It was approved by the Ethics Committee at the West China Hospital, Sichuan University.

MATERIALS AND METHODS

From April 2005 to December 2008, 423 adult patients underwent liver transplantation in our hospital. CNI-based immunosuppression regimens comprising a CNI (cyclosporine A or tacrolimus) + azathioprine + prednisone were delivered to recipients after transplantation. All patients who received a CNI-based regimen for immunosuppression after liver transplantation were potential candidates for this study. Recipients receiving CNI therapy without interruption after liver transplantation, with normal SCr at baseline and detectable microproteinuria in fresh urine were all included in this study. If death occurred within 3 mo posttransplantation or renal dysfunction was caused by non-CNI drugs (such as antibiotics or antivirals), or by other means during follow-up period, the recipients were excluded from the study.

Follow-up protocols were performed once every month for the first 6 mo after liver transplantation, once every 2 mo during months 7-12, and once every 6 mo after 1 year. At each time point, the patient contributed a urine sample which was correlated with the measurements from blood samples. Midstream fresh urine samples were collected and not centrifuged. Measurements of microproteinuria including α1-microglobulin (α1m), β2-microglobulin (β2m), immunoglobulin, microalbumin and transferrin were performed immediately with a Dade Behring array nephelometry system (Dade Behring Inc, USA). SCr (picric acid method) was measured by a Modula clinical chemistry analyzer (Roche Diagnostics, Roche, Switzerland). GFR was measured every 6 mo by the Gates method (PHILIPS Helix SPX-6D SPECT, Holland) which analyzed the blood clearance of ^{99m}Tc-diethylenetriaminepentaacetic acid (DTPA) (5 mCi, from the China Institute of Atomic Energy, radiochemical purity 95%). As consent for a renal biopsy was difficult to obtain, this was performed only when clinically indicated, especially in patients with increasing SCr (an indication of progressive deterioration in renal function).

The actual GFR is considered to be the best overall index of renal function in health and disease[11].Therefore, we chose the actual GFR as the criteria for renal function in this study. The patients were divided into 4 groups according to the mean values of GFR at every 6-mo follow-up: Group 1, GFR declined from baseline by 0%-10%; Group 2, GFR declined from baseline by 11%-20%; Group 3, GFR declined from baseline by 21%-40%; Group 4, GFR declined from baseline by > 40% and/or SCr was increasing.

The normal concentrations of individual proteins present in the urine are illustrated by maximum values[12,13], and are significantly different between different laboratories[12-14]. The normal reference values of microproteinuria provided by Dade Behring, Inc. are: microalbumin < 30 mg/L, α1m < 12.0 mg/L, β2m < 0.20 mg/L, immunoglobulin < 9.6 mg/L, and transferrin < 1.9 mg/L. According to the values determined in 500 Chinese healthy individuals in our laboratory, the normal values used in this study for microalbumin were < 19 mg/L, α1m < 12.5 mg/L, β2m < 0.22 mg/L, immunoglobulin < 20.0 mg/L, and transferrin < 2.2 mg/L.

Statistical analysis was performed by SPSS 13.0 (SPSS Inc., Chicago, IL). Differences in microproteinuria, serum creatinine and BUN were tested with the one way ANOVA test for multiple comparisons. Data were expressed as mean ± SD, or median (range). The Kruskal-Wallis rank sum test (individual comparisons were done by the Wilcoxon rank sum test) and correlation analyses were used in this study. A *P*-value less than 0.05 indicated statistical significance.

RESULTS

A total of 143 liver transplant patients were recruited into this study. Of these, 16 withdrew during the follow-up period. The reasons for withdrawal were infection in 3 patients, acute rejection in 2, use of non-CNI drugs in 4, uncontrolled hypertension in 3, abnormal liver function in 2, and 2 patients died. There were 23 females and 120 male, aged 21-68 years. The grafts and recipients were blood group identical in 127 cases and compatible in 16 cases. The mean follow-up was 32 mo (range 16-36 mo). The primary disease of the 143 recipients included diffuse ischemic intrahepatic biliary stenosis in 8, Caroli disease in 7, Budd-Chiari syndrome with liver cirrhosis in 4, liver cirrhosis after hepatitis B in 56, postoperative liver failure after right lobe hepatectomy caused by hepatic trauma in 4, Wilson disease in 11, α1-antitrypsin deficiency in 2, echinococcus disease of the liver in

Table 1 Demographic data of patients at the point of entry into this study (mean ± SD)

Variable (*n* = 143)	Group 1 (*n* = 102)	Group 2 (*n* = 35)	Group 3 (*n* = 6)	Group 4 (*n* = 0)
Age (yr), median(range)	48.6 (28-66)	49.2 (33-67)	48.5 (21-68)	0
Body mass index (BMI) (kg/m^2)	22.0 ± 2.8	20.5 ± 2.4	21.8 ± 2.9	0
Pre-transplant MELD score	17.8 ± 7.6	16.2 ± 8.4	15.7 ± 9.7	0
BUN (mmol/L)	5.6 ± 2.3	6.0 ± 2.5	5.9 ± 2.8	0
SCr (μmol/L)	54.7 ± 15.8	60.5 ± 20.1	68.7 ± 17.4	0
GFR (mL/min per 1.73 m^2)	105.4 ± 20.5	99.6 ± 15.2	103.3 ± 23.1	0
Dosage (mg/kg per day)				
Cyclosporine A	4.0 ± 1.1	4.6 ± 1.5	5.1 ± 1.2	0
Tacrolimus	0.055 ± 0.03	0.061 ± 0.07	0.057 ± 0.05[a]	0
Trough levels (ng/mL)				
Cyclosporine A	212.4 ± 45.8	243.7 ± 56.2	251.3 ± 61.6	0
Tacrolimus	6.3 ± 0.6	6.7 ± 0.7	7.5 ± 0.9[a]	0
Duration of previous treatment (mo)				
Cyclosporine A	5.8 ± 3.4	6.7 ± 4.6	6.9 ± 3.9	0
Tacrolimus	5.3 ± 2.8	6.2 ± 3.5	6.7 ± 2.5	0
Time post-transplant	7.5 ± 2.2	8.8 ± 2.6	8.2 ± 3.1	0
Microproteinuria (mg/L)				
β2m	0.2 ± 0.1	0.3 ± 0.1	0.5 ± 0.3[a]	0
α1m	14.2 ± 10.6	18.5 ± 17.3	24.8 ± 20.1[a]	0
Microalbumin	28.6 ± 19.3	37.7 ± 28.1	40.1 ± 30.5	0
Immunoglobulin	27.4 ± 8.3	25.1 ± 11.4	29.3 ± 15.6	0
Transferrin	2.8 ± 0.7	2.4 ± 1.6	3.1 ± 1.4[a]	0

[a]$P < 0.05$, *vs* group 1. Group 1: GFR declined from baseline by 0%-10%; Group 2: GFR declined from baseline by 11%-20%; Group 3: GFR declined from baseline by 21%-40%; Group 4: GFR declined from baseline by > 40% and/or SCr was increasing.

Table 2 Mean values of every follow-up time point in the study groups (mean ± SD)

Variable (*n* = 143)	Group 1 (*n* = 73)	Group 2 (*n* = 40)	Group 3 (*n* = 30)	Group 4 (*n* = 5)
GFR (mL/min per 1.73 m^2)	97.4 ± 12.7	85.6 ± 17.9	62.3 ± 20.5	49.6 ± 20.2
The declining percentage of GFR from baseline (%)	7.3 ± 2.6	16.7 ± 10.1[a,c]	32.5 ± 12.9[a,c]	52.4 ± 20.8[a,c]
SCr (μmol/L)	76.3 ± 16.2	83.7 ± 15.4	90.3 ± 19.8	173.7 ± 28.5[a,c]
BUN (mmol/L)	5.8 ± 1.7	6.3 ± 1.2	6.9 ± 1.5	11.2 ± 2.6[a,c]
Dosage (mg/kg per day)				
Cyclosporine A	2.5 ± 0.8	3.0 ± 1.0	3.3 ± 0.9	3.8 ± 1.1[a]
Tacrolimus	0.049 ± 0.03	0.052 ± 0.07	0.054 ± 0.05	0.058 ± 0.03[a]
Trough levels				
Cyclosporine A (ng/mL)	145.2 ± 30.5	154.8 ± 25.6	165.3 ± 39.4	183.2 ± 31.2[a]
Tacrolimus (ng/mL)	5.1 ± 0.8	5.5 ± 1.2	6.2 ± 1.0[a]	6.8 ± 1.3[a,c]
Diabetes (%)	9.2	10.5	9.3	11.1
Hypertension (%)	38.5	34.2	37.2	33.3
Follow-up time, mean (range, mo)	35.6 (23-36)	33.7 (24-36)	30.4 (19-36)	27.8 (16-36)
Microproteinuria				
β2m (mg/L)	0.2 ± 0.1	0.4 ± 0.2[a]	1.0 ± 1.3[a,c]	4.2 ± 2.5[a,c]
α1m (mg/L)	22.6 ± 21.1	38.9 ± 25.4[a]	42.3 ± 35.9[a,c]	65.1 ± 30.4[a,c]
Microalbumin (mg/L)	46.9 ± 26.2	83.6 ± 55.5[a]	70.9 ± 33.8	45.4 ± 32.9
Immunoglobulin (mg/L)	23.9 ± 14.6	21.5 ± 29.1	37.3 ± 26.4	33.5 ± 29.1
Transferrin (mg/L)	3.2 ± 10.2	10.8 ± 27.8	8.2 ± 15.9	8.1 ± 12.4

[a]$P < 0.05$, *vs* group 1; [c]$P < 0.05$, *vs* group 2. Group 1: GFR declined from baseline by 0%-10%; Group 2: GFR declined from baseline by 11%-20%; Group 3: GFR declined from baseline by 21%-40%; Group 4: GFR declined from baseline by > 40% and/or SCr was increasing.

4, hepatolithiasis in 3, hepatocellular carcinoma in 38, and cholangiocarcinoma in 6. All hepatocellular cancer patients met the UCSF criteria (a single tumor ⩽ 6.5 cm in diameter, or 2 or 3 tumors, none exceeding 4.5 cm in diameter and whose sum of tumor diameters did not exceed 8 cm). The demographic data of these patients are shown in Table 1. There were 102 (71.3%) recipients in Group 1, 35 (24.5%) in Group 2, 6 (4.2%) in Group 3, and none in Group 4 at baseline. At entry into this study, all recipients had normal levels of BUN, SCr, and GFR with detectable microproteinuria in fresh urine. There were no significant differences in body mass index, or pre-transplant MELD score.

Through measurements of GFR by the blood clearance of ^{99m}Tc-DTPA at entry into the study and at the follow-up visits, we found there was a downward trend in renal function over time, and the reductions in GFR were significantly different across all groups (Table 2). The value of GFR was 97.4 ± 12.7 mL/min per 1.73 m^2 in Group 1 (decreased 7.3% ± 2.6% from baseline), and 85.6 ± 17.9 mL/min per 1.73 m^2 in Group 2 ($P < 0.001$ *vs* Group 1) (decreased 16.7% ± 10.1% from baseline), and

62.3 ± 20.5 mL/min per 1.73 m^2 in Group 3 ($P < 0.001$ *vs* Group 1; $P < 0.001$ *vs* Group 2) (decreased 32.5% ± 12.9% from baseline). In Group 4, the value of GFR was 49.6 ± 13.2 mL/min per 1.73 m^2 ($P < 0.001$ *vs* Group 1; $P < 0.001$ *vs* Group 2; $P = 0.002$ *vs* Group 3) (decreased 52.4% ± 20.8% from baseline). The SCr significantly increased only in Group 4 (173.7 ± 28.5 umol/L) when compared with the other groups ($P = 0.017$ *vs* Group 1, $P = 0.021$ *vs* Group 2, $P = 0.035$ *vs* Group 3). Similar trends were found for BUN. However, the urinary β2m ($P < 0.01$) and α1m ($P = 0.043$) were significantly different between the 4 groups of patients. This study also indicated that microalbumin, immunoglobulin and transferrin had no significant differences in the 4 groups of patients.

In Group 1, the mean level of urinary α1m at entry and at subsequent follow-up visits was 22.6 ± 21.1 mg/L, which was significantly lower than that in Group 2 (38.9 ± 25.4 mg/L, $P < 0.001$), and in Group 3 (42.3 ± 35.9 mg/L, $P < 0.001$). In Group 4, the level of urinary α1m (65.1 ± 30.4 mg/L) was significantly higher than that of all the other groups ($P < 0.001$ *vs* Group 1; $P < 0.001$ *vs* Group 2; $P = 0.002$ *vs* Group 3). The findings in this study suggested that the increase in urinary α1m occurred long before the elevation of SCr or BUN, and the reductions in GFR were closely correlated with the increases in α1m ($r^2 = -0.728$, $P < 0.001$). The urinary α1m, as a marker of tubular damage, was sensitive in assessing subtle changes in renal function caused by CNI nephrotoxicity.

The mean level of urinary β2m during follow-up visits was 0.2 ± 0.1 mg/L in Group 1, 0.4 ± 0.2 mg/L in Group 2 ($P = 0.041$ *vs* Group1) and 1.0 ± 1.3 mg/L in Group 3 patients ($P < 0.001$ *vs* Group 1; $P < 0.001$ *vs* Group 2). The level of urinary β2m in Group 4 patients (4.2 ± 2.5 mg/L) was further increased compared to other subgroups ($P < 0.001$ *vs* Group 1; $P < 0.001$ *vs* Group 2; $P < 0.001$ *vs* Group 3). The urinary β2m concentration gradually increased from Group 1 to Group 4. The results of this study showed that the increase in urinary β2m, as a marker of tubular epithelial cell dysfunction, occurred long before the elevation of SCr or BUN, and was also closely correlated with the reduction in GFR ($r^2 = -0.787$, $P < 0.001$). The urinary β2m was also sensitive for detecting subtle changes in renal function in patients exposed to CNI-based immunosuppression regimens. Additionally, monitoring both β2m and α1m could improve the sensitivity and specificity of measurement of CNI-related nephrotoxicity.

DISCUSSION

The tremendous success of CNIs in reducing acute rejection episodes and early immunologic graft injury has not been accompanied by a benefit in long-term recipient survival[15,16]. CNI nephrotoxicity in liver transplantation is a significant concern and appears to be progressive over time when CNI exposure is maintained[17]. Microproteinuria has been used as an early marker of nephrotoxicity to detect small changes in the function of tubular epithelial cells in many pathological conditions[10]. The persistence of microproteinuria may result from drug toxicity or pretransplant renal diseases after liver transplantation. Therefore, recipients with normal serum creatinine at baseline and detectable microproteinuria were selected as subjects in this study.

Follow-up data of this study demonstrated that there was a downward trend in renal function over time, with the persistence of microproteinuria. The urinary concentration of β2m and α1m significantly increased with the subtle change in renal function in all study groups, but the levels of SCr and BUN significantly increased only when renal function was severely reduced by CNI nephrotoxicity (in Group 4, renal function declined 52.4% ± 20.8 % from baseline). A similar study also found microproteinuria occurred long before the elevation of SCr[18]. The subsequent reductions in GFR were closely correlated with elevated α1m ($r^2 = -0.728$, $P < 0.001$) and β2m ($r^2 = -0.787$, $P < 0.001$) in the study groups. The results of this study were similar to another report[19], indicating that tubular epithelial dysfunction defined by elevation of tubular injury biomarkers (β2m or α1m) was very common when CNI exposure was maintained. Additionally, as β2m is unstable in fresh urine, fewer patients were found to have β2m in the urine than α1m in this study. This problem can partly be overcome by maintaining the urine pH value (by adding basic buffer to the urine) to prevent the degradation of β2m. This study suggested that urinary β2m and α1m are sensitive urinary markers for detecting CNI-related nephrotoxicity in liver transplant recipients.

In conclusion, monitoring of patients with SCr requires a higher laboratory effort and the use of gender-specific cut-off values. Measurement of microproteinuria is easily available, non-expensive, and convenient in daily clinical practice. The urinary β2m or α1m can be used as an early, sensitive and simple diagnostic indicator for detecting CNI-related renal dysfunction. Furthermore, it should be used as the screening method after liver transplantation to prevent the progressive deterioration of subclinical renal dysfunction.

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COMMENTS

Background

Deterioration of renal function with calcineurin inhibitor (CNI) therapy has been widely reported in liver transplant recipients. Monitoring of renal function, however, is still dependent on somewhat old technologies: serum creatinine (SCr), blood urea nitrogen (BUN), total urine output. Whether microproteinuria could be used as an early and sensitive indicator to monitor CNI-related nephrotoxicity in liver transplant recipients has not been unequivocally addressed.

Research frontiers

Measurement of SCr, BUN, and total urine output are particularly problematic, as they do not have sufficient specificity, sensitivity, or accuracy to allow appropriate and timely prevention of the deterioration of renal function. In this field, the research goal is to identify more sensitive indicators in the early diagnosis of CNI-related nephrotoxicity in liver transplant recipients.

Innovations and breakthroughs

Recent reports have highlighted that nephrotoxicity of CNIs contributes to renal function deterioration in liver transplant recipients. This is the first study to investigate whether microproteinuria could be used as an early and sensitive indicator to monitor CNI-related nephrotoxicity in liver transplant recipients.

Applications

By using β2-microglobulin (β2m) and α1-microglobulin (α1m) as markers for early diagnosis of CNI-related nephrotoxicity, this study may present a future screening method for preventing the progression of CNI-related renal dysfunction in liver transplant recipients.

Terminology

Microproteinuria: it is a hallmark of the early changes in glomerular and proximal tubular function and includes α1m, β2m, immunoglobulin, microalbumin and transferrin.

Peer review

Microproteinuria was studied in these study groups at entry and at subsequent follow-up visits and was correlated with glomerular filtration rate. It revealed that microproteinuria could be used as an early and sensitive indicator to monitor CNI-related nephrotoxicity in liver transplant recipients. This manuscript is very interesting and may present a future screening method to prevent the progression of CNI-related subclinical renal dysfunction after liver transplantation.

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CASE REPORT

Surgical treatment of locally advanced anal cancer after male-to-female sex reassignment surgery

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Abstract

We present a case of a transsexual patient who underwent a partial pelvectomy and genital reconstruction for anal cancer after chemoradiation. This is the first case in literature reporting on the occurrence of anal cancer after male-to-female sex reassignment surgery. We describe the surgical approach presenting our technique to avoid postoperative complications and preserve the sexual reassignment.

Key words: Sex reassignment; Pelvic surgery; Anal cancer

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INTRODUCTION

Pelvic surgery after male to female sex reassignment implies a particular surgical approach: firstly, anatomic variations during resection can be considerable; secondly, although genital reconstruction is an essential target for patients, it is a difficult operation especially after chemoradiation. We present a case of a transsexual patient who underwent a partial pelvectomy and genital reconstruction for anal cancer.

CASE REPORT

A 63 year-old patient presented with rectal bleeding and anal pain. In 1982, she was submitted to male-to-female sex reassignment surgery according to the technique described by Meyer *et al*[1] consisting of penile disassembly followed by vaginoplasty using all penile components except the corpora cavernosa.

Physical examination on January 2006 revealed an anal ulcer and a biopsy showed evidence of a squamous carcinoma. Clinical staging at diagnosis indicated an involvement of the inguinal lymph nodes (T2N2M0, stage Ⅲ B anal cancer[2]).

Concurrent chemoradiation was administered according to the following scheme: the radiation field included the primary tumour and inguinal lymph nodes; a total of 45 Gy in 25 fractions was administered at the primary tumour and a total of 30.6 Gy at the inguinal fields. Each chemotherapy cycle lasted 38 d and consisted of oxaliplatin 180 mg/m^2 on days 1, 19 and 38 and xeloda 1300 mg/m^2 concurrently to radiotherapy. A radiotherapy boost was also administered either on the primary tumour or the inguinal lymph nodes.

Clinical evaluation six weeks after the completion of chemoradiation showed a complete clinical response. At one year follow-up, CT scan showed local recurrence involving the anal sphincter, neo-vagina and bladder neck. A surgical approach was planned in collaboration with reconstructive surgery in order to preserve sex reassignment.

The surgical approach consisted of two parts.

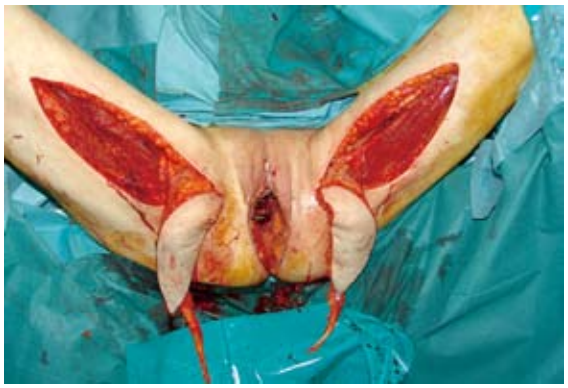

Figure 1 Elliptical skin islands and flaps preparation.

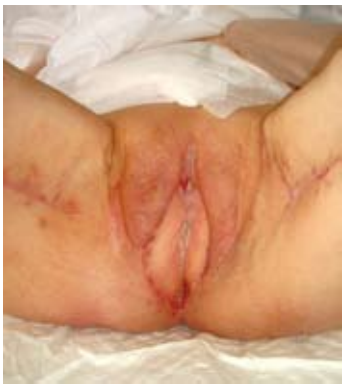

Figure 2 Reconstruction after one month.

During the abdominal portion of the procedure, the rectum was mobilized, and the posterior bladder wall and the seminal vesicles were exposed. During the pelvic portion of the procedure the excision was completed. The perineal skin incision included the neo-vagina and urethra. The rectum and the anus were removed en bloc with the bladder neck, neo-vagina, seminal vesicles and prostate. The bladder neck was sutured, and sovrapubic cystostomy and the colostomy were performed.

Following the excision, a loss of substance of 20 cm × 15 cm resulted. An elliptical skin island up to 15 cm × 6 cm was outlined over the proximal two-thirds of the gracilis muscle of each thigh, according to a technique previously described by our group[3] (Figure 1). The anterior margin of the incision lay on a line drawn between the pubic tubercle and the semitendinosus tendon. The gracilis tendon was identified distally, and the tendinosus insertion divided. The posterior incision was made down to the muscle. The flap was then elevated from distal to proximal on the thigh. The vascular supply of the flap was the medial femoral circumflex artery, which enters the gracilis muscle 7-10 cm below the pubic tubercle. Once the pedicle was identified and preserved, the proximal muscle was dissected. The entire myocutaneous flap was tunnelled through the subcutaneous skin bridge into the vaginal defect and exteriorized through the introitus. The bilateral flaps were sutured to each other at the midline. The neovagina was shaped into a pouch and then inserted into the pelvic space that left after the exenteration. The proximal end of the neovagina was sutured to the introitus. The result after one month is presented in Figure 2.

The postoperative course was complicated by the occurrence of urinary leak, successfully treated with bilateral temporary nephrostomy.

The histology confirmed a locally advanced squamous carcinoma of the anus. Surgical margins were involved. The patient is free of disease at 8 mo follow-up.

DISCUSSION

This is the first case in literature reporting on the occurrence of anal cancer after male-to-female sex reassignment surgery. We tried to achieve two important targets: (1) to avoid postoperative complications of extended surgery after chemoradiation. In fact, perineal reconstruction with gracilis muscle flaps proved very useful in preventing the primary postactinic healing delay. Immediate perineal reconstruction is helpful in avoiding delayed complications due to radiotherapy, namely tissue sclerosis and irreversible damage to irradiated tissues. Gracilis flap transposition ensures a persistent vascular contribution to the irradiated perineal tissues, increasing local oxygenation and leading to more rapid recovery of the surgical wound. In addition to the vascular advantages, the gracilis muscular flap, fixed to the pelvic floor, prevents dead spaces and thus reduces the incidence of seromas. Extended perineal surgery can subvert the architecture of the pelvic floor reducing the sustaining forces and leading to herniation into the perineal space. We would like to point out that the muscular gracilis flap is crucial to compensate for the lack of perineal support after this operation. Once the defect is closed, no further procedure is needed to treat the above mentioned complications in unreconstructed patients, allowing patients to recover quickly and possibly receive adjuvant therapies. (2) to reconstruct the sexual reassignment. This point may not be essential for the surgeon who often assumes that radical surgery is the only target of the operation, but it remains an important issue for patients.

In conclusion, this is the first case in literature reporting on surgical treatment of locally advanced anal cancer after male-to-female sex reassignment surgery. Surgical reconstruction is a reasonable and helpful option to avoid severe complications and preserve sexual reassignment.

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CASE REPORT

Anabolic steroid-induced cardiomyopathy underlying acute liver failure in a young bodybuilder

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Abstract

Heart failure may lead to subclinical circulatory disturbances and remain an unrecognized cause of ischemic liver injury. We present the case of a previously healthy 40-year-old bodybuilder, referred to our Intensive-Care Unit of Hepatology for treatment of severe acute liver failure, with the suspicion of toxic hepatitis associated with anabolic steroid abuse. Despite the absence of symptoms and signs of congestive heart failure at admission, an anabolic steroid-induced dilated cardiomyopathy with a large thrombus in both ventricles was found to be the underlying cause of the liver injury. Treatment for the initially unrecognized heart failure rapidly restored liver function to normal. To our knowledge, this is the first reported case of severe acute liver failure due to an unrecognized anabolic steroid-induced cardiomyopathy. Awareness of this unique presentation will allow for prompt treatment of this potentially fatal cause of liver failure.

Key words: Acute liver failure; Anabolic steroids; Bodybuilder; Cardiomyopathy; Hypoxic hepatitis

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INTRODUCTION

In the absence of a sudden and profound reduction in systemic blood pressure, heart failure is frequently forgotten in the differential diagnosis of acute hepatitis[1]. However, it has long been recognized that ischemic liver injury may follow subclinical circulatory disturbances, particularly in patients with pre-existing passive hepatic congestion, and present as acute hepatitis[2]. Here, we report the first case of severe acute liver failure due to an unrecognized anabolic steroid-induced cardiomyopathy.

CASE REPORT

A 40-year-old male bodybuilder was transferred to our Intensive-Care Unit of Hepatology for treatment of severe acute liver failure. The patient had a history of anabolic steroid abuse over the last 10 years, self-administered in cycles of 6-10 wk, with a 2-3 wk suspension period between cycles. The most frequently used anabolic steroids were: methandrostenolone, stanozolol and oxymetholone (oral); and nandrolone decanoate, testosterone enanthate and trenbolone enanthate (intramuscular). Notably, he used massive doses of all anabolic steroids, including trenbolone enanthate (500-700 mg per week). There was no history of alcohol abuse or acetaminophen intake. He had no family history or past personal history of liver or cardiovascular diseases. The patient was in good physical condition until approximately one month prior to admission, when he experienced increasing fatigue, decreased exercise tolerance and general malaise. Although he stopped exercising and self-administrating the drugs, these symptoms continued to progress and he subsequently developed anorexia, recurrent vomiting, right upper-quadrant abdominal pain and progressive jaundice in the 4 d prior to admission. No history of dyspnea, orthopnea, paroxysmal nocturnal dyspnea or edema could be elicited. At initial evaluation in the patient's local hospital, laboratory

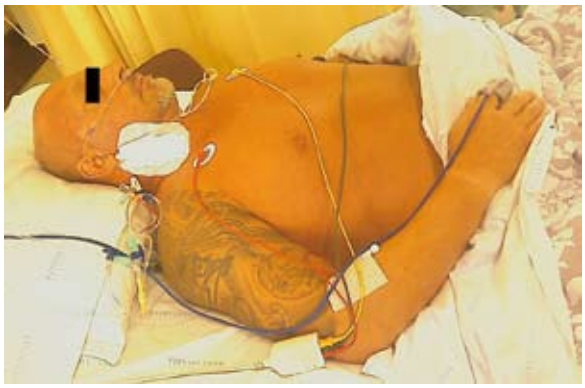

Figure 1 The patient was an athletic young male, with significant jaundice, in no acute distress and able to lie flat.

testing revealed an increase in serum transaminases (aspartate aminotransferase, 7897 IU/L; alanine aminotransferase, 7125 IU/L), coagulopathy (international normalized ratio, 3.3; factor V, 15%), hyperbilirubinemia (total bilirubin 6.8 mg/dL), high ammonia levels (73 umol/L) (normal, 11-32), acute renal failure (creatinine, 3.8 mg/dL), hyponatremia (126 mmol/L) and high lactate dehydrogenase (LDH) level (7140 IU/L). An abdominal ultrasound revealed hepatomegaly and patent flow of the inferior vena cava and hepatic veins. By the end of the first day, the patient was transferred to our Intensive-Care Unit of Hepatology, with the suspicion of anabolic steroid-induced toxic hepatitis.

On transfer, the patient appeared to be an athletic young male in no acute distress and was able to lie flat (Figure 1). Blood pressure was 96/60 mmHg, pulse was 120 beats/min, and respiratory rate was 26 breaths/min. He was conscious with no signs of hepatic encephalopathy. Additional findings on physical examination included generalized jaundice, no evidence of jugular venous distension, clear lung fields and distant heart sounds. He had an enlarged tender liver and no stigmata of chronic liver disease. His extremities were warm, with slight pretibial edema. Laboratory data were similar to those on admission, with markedly depressed values of factor V (15%) and factor Ⅶ (6%). Free testosterone and delta 4-androstenedione concentrations were elevated. Acetaminophen level was undetectable. Serology of known hepatotropic viruses was negative. Antinuclear antibodies were undetectable and serum copper and ceruloplasmin were normal. On chest X-ray, the cardiothoracic index was augmented with clear lung fields. The case was presented to our liver transplantation centre. After correction of hyponatremia with saline, in an attempt to obtain a mildly hyperosmotic state and prevent cerebral edema[3], symptoms and signs of congestive heart failure became evident, with pulmonary congestion and elevated central venous pressure. Echocardiogram showed a dilated cardiomyopathy with an estimated ejection fraction of 15% and a large thrombus in both ventricles. A diagnosis of severe toxic cardiomyopathy associated with anabolic steroids was made after ruling out other causes of non-ischemic dilated cardiomyopathy, including infectious, autoimmune and metabolic causes. With aggressive therapy for cardiac failure with dopamine, dobutamine and watchful diuresis, the patient's liver function improved dramatically. There was a rapid fall in serum transaminases and LDH levels. Four days after admission, the international normalized ratio was < 2.0 and low-molecular weight heparin therapy was started. Serial echocardiograms showed the disappearance of the intraventricular thrombus and improvement in left ventricular function (the fractional shortening increased up to 25%). Coronary angiography disclosed no lesions. After a 16-d hospitalization, the patient was discharged with oral anticoagulation. He his presently in the New York Heart Association functional class Ⅱ, working as a nightclub security agent. However, not enough time has elapsed since treatment to assess full recovery of pathological changes and heart performance.

DISCUSSION

Although the diagnosis of ischemic liver injury can be straightforward in the presence of a clinically evident hemodynamic insult, heart failure may also lead to subclinical circulatory disturbances and remain an unrecognized cause of liver injury[1,2]. Remarkably, in a recent report only around one-half of the patients with ischemic liver injury had been in a state of shock[4]. Isolated cases of unrecognized cardiomyopathy as the cause of severe ischemic liver injury have been reported in the recent literature[5-7]. In these cases, as in our patient, a cardiomyopathy lacking many of the symptoms and signs of congestive heart failure was the cause of the ischemic liver injury, with striking clinical and laboratory evidence of severe liver failure, leading to the possibility of liver transplantation as a rescue therapy. Our patient was an athlete without a history or clinical evidence of heart disease on presentation, and was referred to our Intensive Care Unit of Hepatology for treatment of severe acute liver failure. Several features led to a delay in the diagnosis of the underlying heart disease. Firstly, he was an athletic young male with none of the classic cardiovascular risk factors. Secondly, anabolic steroids are known hepatotoxic drugs[8,9], with only a few reports of severe cardiac toxicity in the literature[10,11]. Thirdly, for unclear reasons, symptoms of congestive heart failure were initially absent; theoretically, the severity of his right-sided heart failure in combination with a low output state may account for this[6]. Finally, cardiac and hepatic diseases share many similar clinical features, such as fatigue, decreased exercise tolerance, hepatomegaly and edema. This case highlights the fact that ischemic injury of the liver should always be considered in the differential diagnosis of acute hepatitis[1]. Retrospectively, some features may have suggested an ischemic rather than a viral or toxic cause of liver injury, including an early massive rise in LDH levels-a ratio of serum alanine aminotransferase to LDH of less than 1.5 may suggest ischemic injury[12]; and a concomitant early rise in the serum creatinine level (additional evidence of end-organ hypoperfusion)[13]. Chest X-ray also revealed

cardiomegaly, despite no evident pulmonary congestion. The rapid fall in serum transaminases after therapy for cardiac failure is characteristic of ischemic liver injury[13].

The medical history of this patient was significant for chronic self-administration of massive doses of anabolic steroids, including trenbolone enanthate, a strictly underground long-active steroid. Although anabolic steroids are associated with significant liver toxicity[8], toxic hepatitis induced by anabolic steroids with predominantly hepatocellular injury is extremely rare[9]. More frequently reported hepatotoxic effects include cholestatic liver injury[8], development of hepatic adenomas[14] and peliosis hepatis[15]. There have been a few reports of severe cardiovascular events associated with anabolic steroid abuse, including cases of severe dilated cardiomyopathy in otherwise young healthy patients[10,11]. Experimental studies[16,17] and non-invasive imaging studies in bodybuilders[18] have demonstrated a dose-dependent impairment of myocardial function after long-term anabolic steroid therapy. It has been proposed that increased fibrosis of the myocardium may be mediated by aldosterone-like effects[10]. The reversibility of such myocardial effects after discontinuation of the drugs is still unknown[18]. A partial recovery of left ventricular function a few months after cessation of anabolic steroid abuse has been reported in two body builders[10].

Anabolic steroid consumption is becoming more widespread and their adverse effects, including cardiovascular and hepatic toxicity, are expected to increase in the years to come[8,10,11]. To the best of our knowledge, this is the first reported case of severe liver failure due to an unrecognized anabolic steroid-induced cardiomyopathy. Awareness of this unique presentation will allow for prompt treatment of this potentially fatal cause of liver failure.

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CASE REPORT

Laparoscopic resection of an adrenal pseudocyst mimicking a retroperitoneal mucinous cystic neoplasm

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Abstract

Adrenal pseudocysts are rare cystic masses that arise within the adrenal gland and are usually non-functional and asymptomatic. Adrenal pseudocysts consist of a fibrous wall without a cellular lining. We report a patient with a 9 cm, left-sided suprarenal cystic mass who presented with abdominal discomfort of 2 years' duration. A 38-year-old woman was referred to our service for evaluation of abdominal discomfort and gastrointestinal symptoms. Routine laboratory tests were within normal limits. An abdominal computed tomography scan showed a 9 cm × 8 cm × 8 cm well-defined cystic lesion displacing the left kidney. Magnetic resonance imaging showed a cystic lesion with low signal intensity on the T1-weighted image and high signal intensity on the T2-weighted image. A laparoscopic left adrenalectomy was performed to diagnose the lesion. The final pathology showed an adrenal pseudocyst without a cellular lining. The patient had no postoperative complications and she was discharged four days after surgery.

Key words: Adrenal gland; Pseudocyst; Laparoscopy; Adrenalectomy

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INTRODUCTION

Adrenal pseudocysts are rare lesions that arise within the adrenal gland, most of which are non-functional and asymptomatic. The majority of adrenal pseudocysts are located in the suprarenal area. Preoperatively, larger cysts might be difficult to distinguish from cysts of renal or pancreatic origin. Adrenal pseudocysts consist of a fibrous wall without a cellular lining. We report a case of a patient with a 9 cm, left-sided suprarenal cystic mass who presented with abdominal discomfort of two years' duration.

CASE REPORT

A 38-year-old woman was admitted to our service with a two-year history of abdominal discomfort, anorexia, and nausea. She had no history of trauma and had undergone a laparoscopic unilateral salpingoophorectomy. A physical examination revealed no palpable abdominal masses and no tenderness in the abdomen. Routine laboratory tests were within normal limits. An abdominal computed tomography scan showed a 9 cm well-defined cystic lesion displacing the left kidney (Figure 1). The cystic lesion appeared to arise from the retroperitoneum. For further evaluation of the cystic lesion, magnetic resonance imaging was carried out and the cystic lesion showed low signal intensity on the T1-weighted image and high signal intensity on the T2-weighted image (Figure 2). There were no findings of suspected malignancy on the radiological image. We decided to perform a laparoscopic resection of the cystic lesion to relieve long-lasting gastrointestinal symptoms and to diagnose the lesion.

The patient was placed in the right lateral decubitus, flexed at the waist. A 12-mm trocar was inserted in the umbilical region *via* an open incision. Three other operating ports were placed along the left costal margin, as shown in Figure 3. The second 10-mm trocar was

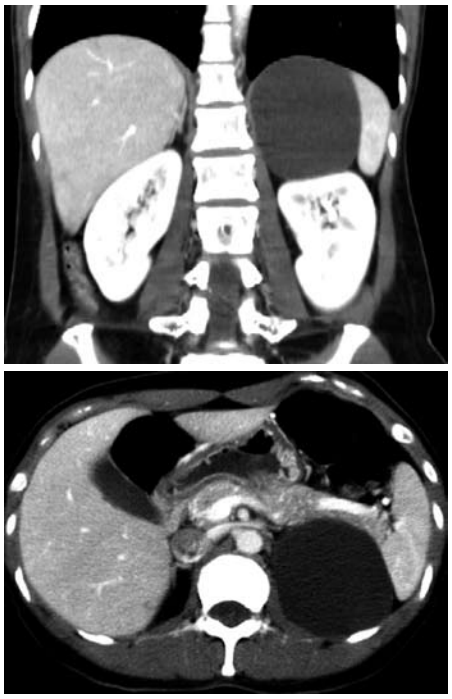

Figure 1 An abdominal computed tomography scan showing a 9 cm well-defined cystic lesion, which abuts the spleen, kidney and pancreas tail.

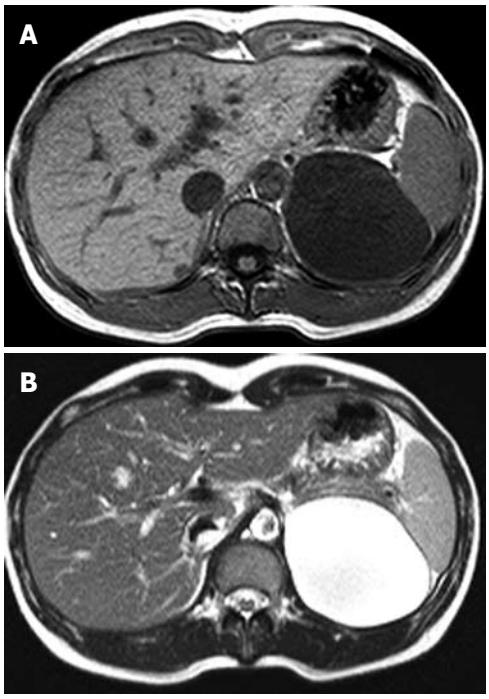

Figure 2 Magnetic resonance imaging of the abdomen. A low signal intensity cystic mass on the T1-weighted image (A) and a high signal intensity cystic mass on the T2-weighted image (B).

introduced on the anterior axillary line. The third 5-mm trocar was introduced on left side of the second trocar and placed under the costal margin. The fourth 5-mm trocar was introduced in the posterior axillary line and was used for a retractor. The splenic flexure of the colon was mobilized from the left paracolic gutter to the inferior pole of the spleen. The splenorenal ligament was divided to allow the spleen and tail of the pancreas to rotate medially, exposing the left retroperitoneum. At that time, a smooth, well-capsulated cystic mass, measuring 9 cm × 8 cm × 8 cm, arising from the left adrenal gland was found. The superior, medial and lateral border of the lesion was first dissected from neighboring tissue with a LigaSure (Valley Lab, Boulder, CO). The dissection proceeded inferiorly to expose and ligate the left adrenal vein. The adrenal vein was identified and divided with a 2.5-mm endoscopic vascular stapler (Ethicon). The adrenal cystic lesion, completely freed, was placed in a surgical bag (Endocatch; Ethicon). The cystic contents were aspirated with a needle and were a thin, yellowish fluid. The specimen was extracted *via* the 10-mm port side by enlarging the incision from 1 to 2.5 cm. The operative time was 110 min and the blood loss was 50 mL. Gross appearance showed a thin-walled, yellowish, 6 cm × 3 cm × 3 cm unilocular adrenal lesion (Figure 4). Histological examination showed an adrenal pseudocyst without an epithelial or endothelial lining (Figure 5). The patient had no postoperative complications and she was discharged four days after surgery. The abdominal pain and gastrointestinal symptoms resolved after surgery.

DISCUSSION

Adrenal cysts are classified as a cystic degeneration of adrenal neoplasms, true cysts, pseudocysts, and infectious cysts[1-3]. Among adrenal cysts, the most common types are epithelial cysts and pseudocysts. True cysts are lined with endothelial or mesothelial cells[4]; however, adrenal pseudocysts are devoid of an epithelial or endothelial lining, arise within the adrenal gland, and are surrounded by a fibrous tissue wall. Infectious cysts are most commonly caused by *Echinococcus*[5].

Adrenal pseudocysts are rare and account for 32% to 80% of all adrenal cysts[3,6-9]. Adrenal pseudocysts are often found incidentally on imaging studies or at the time of autopsy. The majority of adrenal pseudocysts are found because of their size-related symptoms[6-8]. Symptoms include gastrointestinal disturbance, early satiety, and a palpable abdominal mass. Patients can present with acute abdominal findings if intracystic hemorrhage or rupture occurs[10].

The etiology of adrenal pseudocysts is unknown; however, several mechanisms have been proposed to account for their occurrence, including cystic degeneration of a primary adrenal neoplasm, degeneration of a vascular neoplasm, and malformation and hemorrhage of adrenal veins into the adrenal gland[1-3,6,7,11]. The etiology of our patient's pseudocyst was indeterminate.

Due to the wide use of the diagnostic imaging modalities, the detection rate of adrenal cystic lesions is increasing. However, preoperative confirmatory diagnosis of a large adrenal cyst can be very difficult, because of the indistinct boundary with surrounding organs and adhesion to neighboring organs[12].

The differential diagnosis of adrenal pseudocysts includes splenic, hepatic, and renal cysts, as well as mesenteric or retroperitoneal cysts, urachal cysts, and

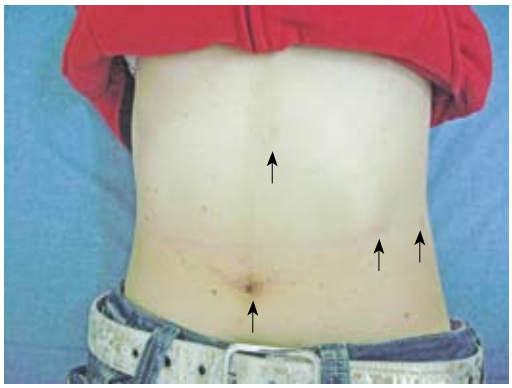

Figure 3 Placement of the laparoscopic trocar and operative ports. A 12-mm trocar was placed in the periumbilical region, and three other operating ports were placed in the abdomen (arrows). The photograph was taken eight months after surgery.

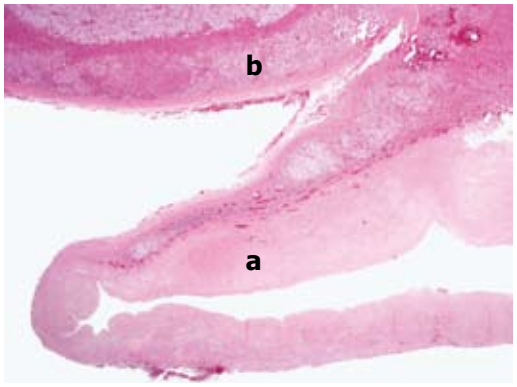

Figure 5 Histopathology showing an adrenal pseudocyst (a) and adrenal tissue remnants on the upper aspect (b). The cyst wall is composed of a thick layer of hyalinized connective tissue without an epithelial or endothelial lining (a). HE staining (Original magnification, × 40).

solid adrenal tumors[12,13]. Adrenal pseudocysts must be differentiated from other benign or malignant lesions originating from the adrenal gland or the kidney unilaterally[14-17]. In addition, an exact diagnosis is clinically important because an adrenal cyst > 6 cm carries an increased risk of adrenal malignancy. The reported incidence of malignancy in adrenal cystic lesions is approximately 7%[18,19].

Typically, radiological findings of adrenal cysts reveal thin walls filled with watery fluid and occasional calcifications[20,21]. Often, the inferior wall of the cyst is concave or flat, conforming to the shape of the upper pole of the renal contour.

On computed tomography scan, the present cyst could not be differentiated from a renal or adrenal lesion. The CT scan was not definitive, therefore an MRI was obtained. The adrenal gland was not visualized by the MRI scan and the cystic mass was thus considered to be of pancreatic rather than adrenal origin. The cystic mass could not be differentiated from a retroperitoneal mucinous cystic neoplasm. We chose the laparoscopic approach because of the time involved, the anticipated minimal blood loss, the acceptable rate of complications, and the excellent postoperative quality of life. There

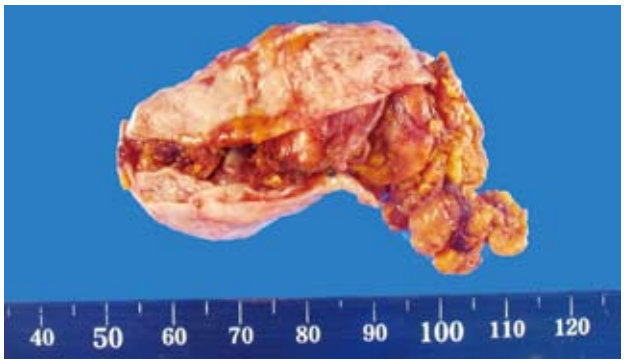

Figure 4 Gross appearance of the 6 cm × 3 cm × 3 cm adrenal cystic lesions. The cystic lesion adheres to adrenal gland tissue.

are few reports of a case of retroperitoneal mucinous cystadenomas treated by laparoscopic surgery[22-24].

During the laparoscopic procedure, we found that the cystic mass had arisen from the left adrenal gland, not the pancreas. We believed that this cystic mass was an adrenal cyst and removed it completely by laparoscopic surgery.

The choice of treatment for adrenal pseudocysts depends on several factors, including endocrine function, symptoms, size, and correct differentiation from an adrenal cyst.

Surgical excision is indicated in the presence of symptoms, suspicion of malignancy, an increase in size, the occurrence of complications, or detection of a functioning adrenal cyst, and can be managed by open surgery or a laparoscopic approach[12,25-27]. Kalady *et al*[28] recommended that adrenal cysts > 6 cm should be approached using an open procedure because of concerns about potential malignancy. In the case of giant pseudocysts, most authors prefer an open adrenalectomy, because the laparoscopic approach is not sufficient to control such large masses with active internal bleeding[29]. However, several reports demonstrate that laparoscopic adrenalectomy for large (> 6 cm) adrenal lesions is surgically feasible and can be applied for any adrenal disease, including benign and potentially malignant lesions[25,30-32].

This case was sufficiently instructive to point out that adrenal cystic lesions can be radiologically mistaken for renal cysts or cystic neoplasms.

This is a rare report of an adrenal pseudocyst mimicking a retroperitoneal mucinous cystic neoplasm, which was treated by laparoscopic resection. Although adrenal pseudocysts might be misdiagnosed as retroperitoneal mucinous cystic neoplasms, a laparoscopic approach to cystic lesions in the suprarenal area should be the initial choice.

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CASE REPORT

Severe acute cholestatic hepatitis of unknown etiology successfully treated with the Chinese herbal medicine Inchinko-to (TJ-135)

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Abstract

Severe acute hepatitis of unknown etiology is difficult to treat and often progresses to subacute fulminant hepatitis or late-onset hepatic failure. A 45-year-old well-nourished, healthy man had progressive fatigue and his liver function tests showed severe liver dysfunction. The etiology of sever acute cholestatic hepatitis was unknown. The liver function tests normalized gradually, which excluded high persistent total bilirubin after starting on predonine. A liver biopsy showed chronic active hepatitis with mild fibrosis (A2, F1). Oral Inchinko-to, a Chinese herbal medicine, at 7.5 g daily was prescribed. The treatment was effective with no adverse effects. We present a successfully treated case and discuss hepatoprotective and choleretic effects of Inchinko-to.

Key words: Acute cholestatic hepatitis; Etiology; Inchinko-to; Herbal medicine

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Ohwada S, Kobayashi I, Harasawa N, Tsuda K, Inui Y. Severe acute cholestatic hepatitis of unknown etiology successfully treated with the Chinese herbal medicine Inchinko-to (TJ-135). *World J Gastroenterol* 2009; 15(23): 2927-2929 Available from: URL: http://www.wjgnet.com/1007-9327/15/2927.asp DOI: http://dx.doi.org/10.3748/wjg.15.2927

INTRODUCTION

Treatment of severe acute hepatitis of unknown etiology is difficult, and the disease often progresses to subacute fulminant hepatitis or late-onset hepatic failure[1]. Several studies have reported on the effectiveness of Inchinko-to, a Chinese herbal medicine used to treat liver disorders and jaundice in Japan[2-5]. Successful treatment of acute hepatic failure of unknown etiology with Inchinko-to has been reported[6].

We treated a patient who suffered from severe acute cholestatic hepatitis of unknown etiology with Inchinko-to. The treatment was effective with no adverse effects. We report and discuss the case.

CASE REPORT

The patient was a 45-year-old man, who had fatigue, vomiting, slight fever, and noticed that his urine had been darker than usual for 1 mo before visiting the clinic. He began to suffer from severe fatigue and loss of appetite, and thus visited the clinic. On physical examination, his skin was dark yellow, without a palpable liver. Liver function tests revealed severe liver dysfunction (Table 1). He was a well-nourished, healthy man with a history of duodenal ulcer. He did not drink alcohol and had no history of blood or blood products transfusion or the use of any drugs, including herbal medicine. He was exposed to no known environmental hazards. His older brother had non-alcoholic steatohepatitis and underwent surgery for rectal carcinoma. Abdominal ultrasonography showed a severely swollen gallbladder and normal intra- and extrahepatic bile ducts; the liver shape and architecture were normal. Upper endoscopy showed an edematous narrowing at the post-bulbar portion of the duodenum and a duodenal ulcer scar in the duodenal bulb.

He was admitted to the hospital. Ursodeoxycholic acid (UDCA) was started at 600 mg daily. Tests for hepatitis A, B, C, and E viruses, Epstein-Barr virus and cytomegalovirus were all negative. Autoantibodies were negative, including anti-mitochondrial, smooth muscle, antinuclear, and perinuclear anti-neutrophil cytoplasmic antibody. Gamma globulin was within the normal limits. The hemolytic complement activity (CH50) was elevated slightly. Abdominal computed tomography showed a severely swollen gallbladder, normal intra- and extrahepatic bile ducts, and normal liver shape and architecture (Figure 1, top). Magnetic resonance imaging showed an edematous,

Table 1 Laboratory data on admission

White blood cells (/μL)	4600	Alfa1 globulin (%)	4.2
Red blood cells (/μL)	493	Alfa2 globulin (%)	7.2
Hemoglobin (g/dL)	15.1	Beta globulin (%)	11.2
Hematocrit (%)	46	Gamma globulin (%)	15.3
Platelets (/μL)	16.7×10^4	CH50 (OU/mL)	52.4
Total protein (g/dL)	5.8	Anti-nuclear antibody	-
Albumin (g/dL)	3.5		
Total bilirubin (mg/dL)	6	P-ANCA	-
Direct bilirubin (mg/dL)	4.9	Anti-mitochondrial	-
AST (IU/L)	810	antibody	
ALT (IU/L)	1239	HA-IgM	-
LDH (IU/L)	418	HBs Ag	-
Alkaline phosphatase	687	HBs Ab	-
(IU/L)		HBe Ag	-
rGTP (IU/L)	305	HCV Ab	-
Prothrombin time (%)	92.9	HEV-IgM	-
Activated partial	29.2	HEV-IgG	-
thromboplastin time (s)		EBV-VCM	-
C-reactive protein (mg/dL)	1	CMV-IgM	-
NH_3 (mcg/dL)	70		

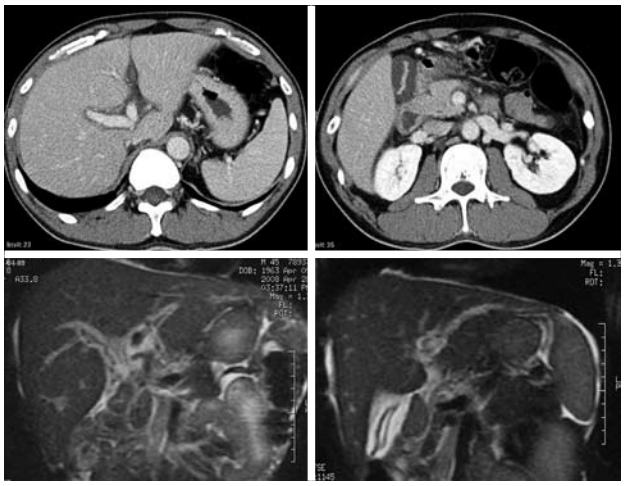

Figure 1 Abdominal CTs and MRIs on admission. Abdominal CTs showing normal liver shape and architecture, normal intra-and extrahepatic bile ducts, and a severely swollen gallbladder (top); MRIs showing a normal liver, bile duct, an edematous, thickened gallbladder wall, and periportal edema (bottom).

thickened gallbladder wall, periportal edema, slight ascites, and a normal liver, bile duct, and pancreatic duct (Figure 1, bottom). Based on these findings, the patient was diagnosed with severe acute cholestatic hepatitis of unknown etiology. The total bilirubin level continued to rise, and so the patient was started on predonine 20 mg daily on 7 May 2008. The serum aspartate aminotransferase, alanine aminotransferase, and lactate dehydrogenase levels normalized gradually, while the total bilirubin remained high. Oral Inchinko-to at 7.5 g daily was prescribed on 17 May 2008. He was weaned from predonine. Subsequently, the total bilirubin level began to decrease (Figure 2). A liver biopsy showed chronic active hepatitis with mild fibrosis (A2, F1).

The patient was discharged. He is currently well at 12 mo after UDCA and Inchinko-to treatment.

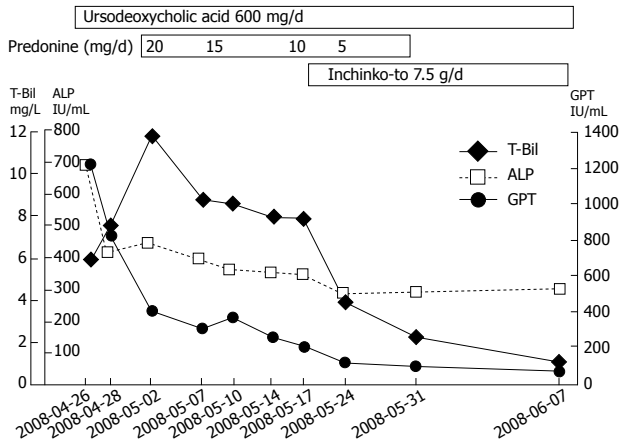

Figure 2 Clinical course of the patient.

DISCUSSION

Without evidence of a virus infection, autoimmune disease, alcohol or drug use, blood or blood products administration, and environmental hazards, the patient was considered to have severe acute cholestatic hepatitis of unknown etiology. Initially, predonine was prescribed for suspected autoimmune hepatitis with a high total bilirubin level[7]. The increasing total bilirubin was thought to suggest progression to subacute fulminant hepatitis[1]. Inchinko-to, a Chinese herbal medicine, has been used clinically for various liver diseases in Japan and China[2-5].

Recent experimental studies have clarified the molecular mechanism of hepatoprotective and choleretic effects of Inchinko-to and its ingredients, and have provided a good rationale for its clinical application to a wide variety of liver diseases, although there is no sound evidence with prospective randomized clinical studies[8-13]. The ingredients of Inchinko-to are genipin, 6,7-dimethylesculetin, capillin, capillene, and capillarisin[2,14,15]. Therefore, we decided to give Inchinko-to to our patient who suffered from severe acute cholestatic hepatitis of unknown etiology. The treatment was effective with no adverse effects.

The death of liver cells in hepatitis is thought to involve apoptosis[16]. Fas/FasL-mediated cytotoxicity is critical to hepatic injury, particularly fulminant hepatitis[17]. Genipin markedly suppressed liver apoptosis/injury in a lethal fulminant hepatitis model[9] and improved acute liver dysfunction by the suppression of tumor necrosis factor-α production[10]. In addition, capillin and capillene inhibit liver cell apoptosis induced by transforming growth factor[8].

Cholestasis results in hepatic and systemic accumulation of potentially toxic bile acids, resulting in liver damage and jaundice[18]. The altered expression of specific hepatocellular transport systems and profound changes in the cytoskeleton of the hepatocytes are associated with cholestatic liver disease[18]. In particular, the impairment of canalicular transport systems has a major role in the pathogenesis of acquired forms of intrahepatic cholestasis[18]. Inchinko-to exerts potent choleretic effects *via* a bile-acid-independent mechanism of multidrug-resistance-associated protein 2 mediation and glutathione content modulation[11-13] by genipin, and of constitutive

androstane receptor activation by 6,7-dimethylesculetin[12]. The marked reduction in bilirubin level after Inchinko-to administration in our case suggested that Inchinko-to and its ingredients stimulated and restored the impaired canalicular transport systems.

UDCA was also used in this case and it has been reported to be effective in cases of chronic liver disease[19] and prolonged cholestasis of acute hepatitis[20]. The therapeutic benefit of UDCA in the treatment of cholestasis may result from a combination of cytoprotective, antiapoptotic, immunomodulatory, and choleretic effects[21]. The choleretic effects of UDCA are medicated by up-regulation of canalicular transporter protein levels[22], and inhibit apoptosis by modulating mitochondrial function[23]. No apparent effect was observed on liver function tests in our case soon after the administration of UDCA. Therefore, the combination of Inchinko-to and UDCA possibly worked synergistically in improving the acute cholestatic hepatitis in this case.

Finally, our success in treating a patient with acute cholestatic hepatitis of unknown etiology supports a previous study[6], and suggests its efficacy in treating such liver disease. Our findings warrant a further clinical trial.

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CASE REPORT

A case of hepatic angiomyolipoma difficult to distinguish from hepatocellular carcinoma

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Abstract

We report a case of hepatic angiomyolipoma with uncommon clinical features. A 56-year-old man presented with a hepatic tumor in the caudate lobe. The tumor was hypoechoic on ultrasonography, showed early-phase hyperattenuation on enhanced computed tomography and did not absorb iron on superparamagnetic iron oxide-enhanced magnetic resonance imaging. Hepatocellular carcinoma was highly suspected, and the patient underwent hepatic resection. Histologically, the tumor was mainly composed of smooth muscle cells and contained small amounts of adipose cells and blood vessels. On immunohistochemical staining, the smooth muscle cells were positive for a melanocytic cell-specific monoclonal antibody. In cases with uncommon features of angiomyolipoma, it is quite difficult to distinguish angiomyolipoma from hepatocellular carcinoma.

Key words: Adipose cell; Hepatic angiomyolipoma; Hepatocellular carcinoma; HMB-45; Smooth muscle cell

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Takahara M, Miyake Y, Matsumoto K, Kawai D, Kaji E, Toyokawa T, Nakatsu M, Ando M, Hirohata M. A case of hepatic angiomyolipoma difficult to distinguish from hepatocellular carcinoma. *World J Gastroenterol* 2009; 15(23): 2930-2932 Available from: URL: http://www.wjgnet.com/1007-9327/15/2930.asp DOI: http://dx.doi.org/10.3748/wjg.15.2930

INTRODUCTION

Hepatic angiomyolipoma (HAML) was first reported by Ishak[1]. It is a rare benign tumor which is composed of a heterogeneous mixture of adipose cells, smooth muscle cells and vessels, and can be treated conservatively if spontaneous hemorrhage or malignant change does not occur[2-4]. The radiological features of HAML depend on the relative proportions of adipose cells[5]. For this reason, preoperative diagnosis of HAML is occasionally difficult, and it is not easy to differentiate from hepatocellular carcinoma (HCC). Herein, we report a case of HAML which was difficult to differentiate from HCC.

CASE REPORT

A 56-year-old Japanese man was admitted to our hospital for further examination of a liver tumor in the caudate lobe. He had no history of liver disease or hepatitis and did not drink heavily. Hepatitis B surface antigen and anti-hepatitis C antibody were negative. Serum levels of transaminase, α-fetoprotein and des-γ-carboxy prothrombin were within the normal range. On ultrasonography, the tumor was hypoechoic (Figure 1A). Enhanced computed tomography (CT) showed a hepatic mass with early-phase hyperattenuation and late-phase hypoattenuation, measuring 4.2 cm × 4.0 cm in the caudate lobe (Figure 1B and C). Magnetic resonance imaging (MRI) revealed hypointensity on T1-weighted images, hyperintensity on T2-weighted images and hyperintensity on diffusion weighted images (Figure 1D-F). The tumor did not absorb iron on superparamagnetic iron oxide-enhanced (SPIO) MRI

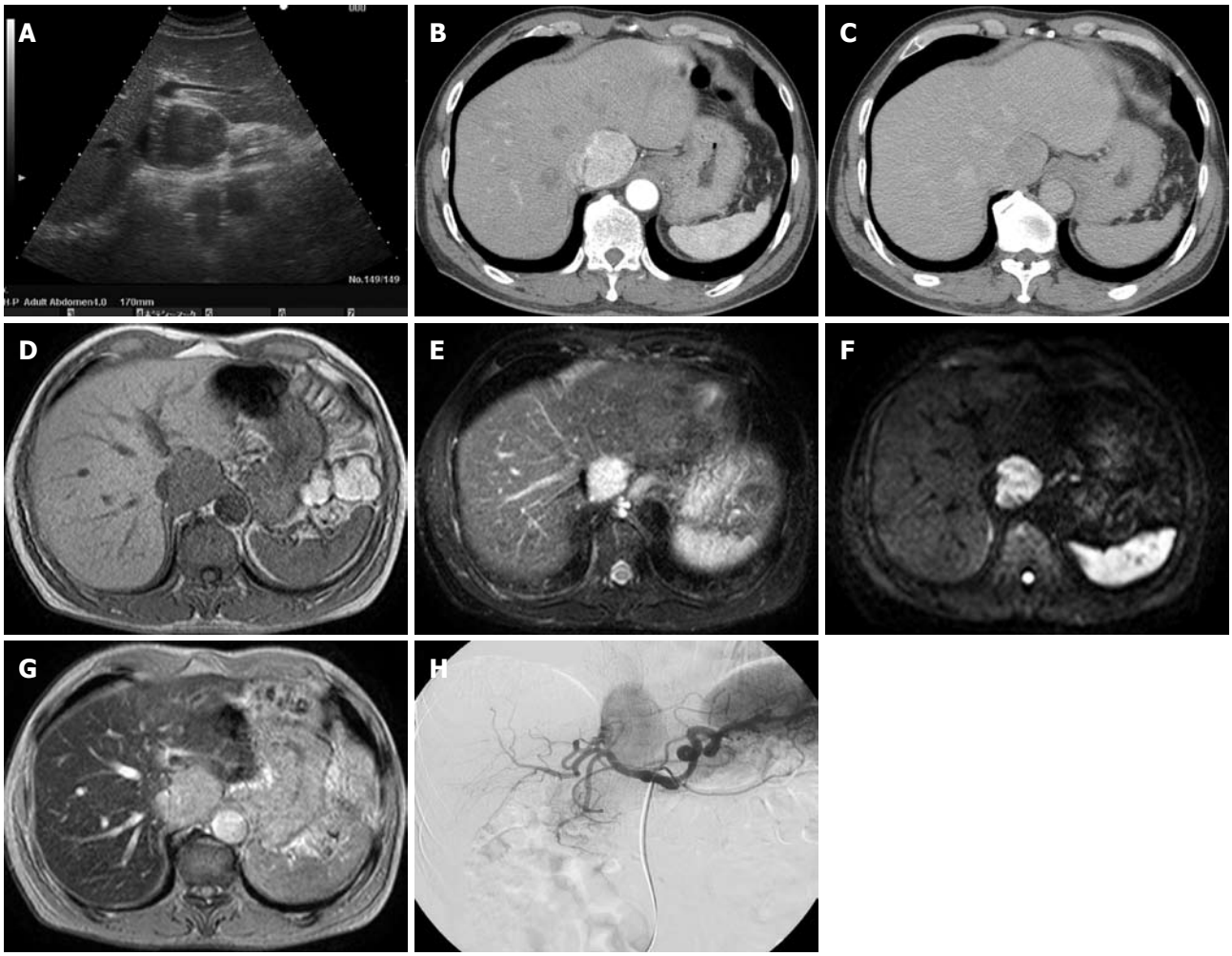

Figure 1 Images. A: The tumor was hypoechoic on ultrasonography, measuring 4.2 cm × 4.0 cm; B-C: Enhanced computed tomography showed a tumor with early-phase hyperattenuation and late-phase hypoattenuation; D-F: Magnetic resonance imaging showed a tumor with hypointensity on T1-weighted, hyperintensity on T2-weighted images and hyperintensity on diffusion weighted images; G: The tumor did not absorb iron on superparamagnetic iron oxide-enhanced MRI; H: On angiography, the tumor was shown as a circumscribed hypervascular mass.

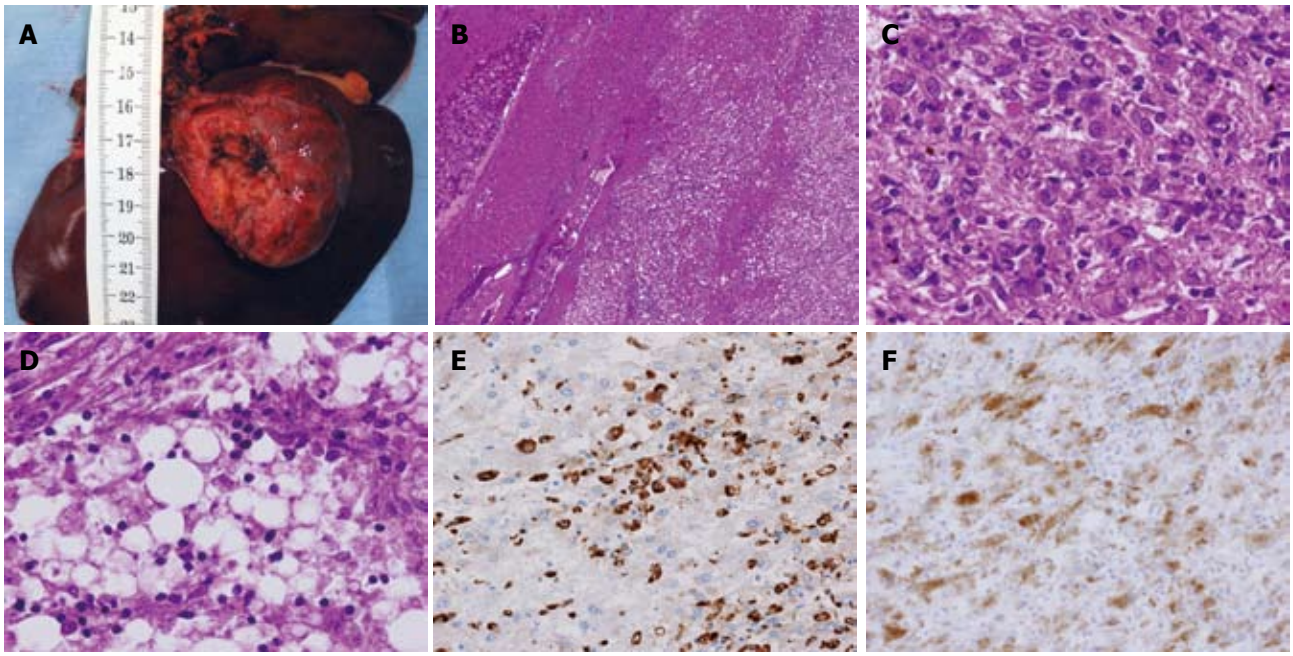

Figure 2 HAML. A: The tumor occupied a large area of the caudate lobe; B-D: The histological features of the tumor showed that it was mainly composed of smooth muscle cells (B: HE stain, × 4; C: HE stain, × 40) and a small number of adipose cells (D: HE stain, × 40); E, F: Immunohistochemically, the tumor was positive for CD68 (E: CD68, × 20), and HMB-45 (F: HMB-45, × 100).

(Figure 1G). On angiography, the tumor was shown as a circumscribed hypervascular mass (Figure 1H). HCC was highly suspected on radiological imaging. Resection of the left lobe with the tumor in the caudate lobe was performed. The tumor measured 6.0 cm in diameter, and the surface of the tumor was gray and white. Histologically, the tumor was mainly composed of smooth muscle cells and contained small amounts of adipose cells and blood vessels (Figure 2A-D). On immunohistochemical staining, the tumor was negative for desmin and S-100, but positive for actin and CD68, and the smooth muscle cells were positive for a melanocytic cell-specific monoclonal antibody (HMB-45) (Figure 2E and F). This tumor was diagnosed as HAML.

DISCUSSION

Recently, the concept of perivascular epithelioid cell tumor (PEComa) which was proposed by Bonetti et al in 1992 has gained wide acceptance[6]. PEComa is defined as a mesenchymal tumor composed of histologically and immunohistochemically distinctive perivascular epithelioid cells. Immunohistochemically, the tumors are consistently immunoreactive for HMB-45, a monoclonal antibody for melanoma. AML is considered a part of PEComa[7]. Smooth muscle cells of HAML stain positively for HMB-45. This finding is useful for the diagnosis of HAML, because liver tumors other than angiomyolipoma are negative for HMB-45[8].

In radiological diagnosis, HAML typically shows high echo on ultrasonography, early-phase hyperattenuation on enhanced computed tomography, hyperintensity on T2-weighted magnetic resonance imaging and a circumscribed hypervascular mass on angiography[9]. The soft tissue component (smooth muscle cells and vessels) is considered to be enhanced by the intravenous administration of contrast material[10]. However, the imaging features of HAML vary because of variations in the proportion of adipose cells, smooth muscle cells and vessels. In particular, the number of adipose cells varies between 10% and 90%[11]. HAML consisting of a small number of adipose cells shows low echo on ultrasonography and early-phase hyperattenuation on enhanced CT. These findings are similar to the imaging features of HCC[12]. On the other hand, there are no reports regarding the radiological features of SPIO MRI on HAML. SPIO contrast material, which is taken up by the reticuloendothelial system and depresses the signal of normal liver at T2-weighted imaging, is useful for the detection of hepatic tumors[13]. In our case, HAML was positive for CD68 stain, which is a Kupffer cell-related marker; however the signal at T2-weighted imaging on SPIO MRI was depressed similar to HCC. Thus, SPIO-MRI was not useful for differentiating HAML from HCC in our case.

HAML shows various histological patterns. According to the line of differentiation and the predominance of tissue components, the tumors are subcategorized into mixed, lipomatous ($\geqslant$ 70% fat), myomatous ($\leqslant$ 10% fat), and angiomatous types. The mixed type is the most common, but tumors with a small number of adipose cells such as the myomatous type, which are rare, show widely variable patterns in morphology[14]. In this study, we classified the HAML in our case as the myomatous type.

In conclusion, HAML is a benign tumor and requires no surgical treatment. However, the diagnosis is difficult because it has various histological patterns. In the myomatous type, the radiological findings including SPIO-MRO are similar to those of HCC. Hepatic tumors without obvious risk factors for HCC should be distinguished from HAML.

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Medline-based bibliometric analysis of gastroenterology journals between 2001 and 2007

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Abstract

AIM: To analyze the MEDLINE-indexed publications in gastroenterology specialty journals from 2001 to 2007. Special attention was paid to specific types of articles, the number of publications for individual authors and the author count in each journal.

METHODS: The bibliographic entries of papers belonging to journals listed under the subject heading of "gastroenterology" were downloaded from MEDLINE on the PubMed web site. The analysis was limited to journal articles published between January 1, 2001 and December 31, 2007. The analytical dimensions of an article included journal, publication year, publication type, and author name (the last name and initials).

RESULTS: According to MEDLINE, 81561 articles were published in 91 gastroenterology journals from 2001 to 2007. The number of articles increased from 9447 in 2001 to 13340 in 2007. Only 12 journals had more than 2000 articles indexed in MEDLINE. The "*World Journal of Gastroenterology*" had the largest number of publications (5684 articles), followed by "*Hepato-Gastroenterology*" (3036) and "*Gastrointestinal Endoscopy*" (3005). Of all the articles published, reviews accounted for 17.2% and case reports for 15.4%. Only 3739 randomized controlled trials (4.6% of all articles) were published and their annual number increased from 442 in 2001 to 572 in 2007. Among 141741 author names appearing in the articles of gastroenterology journals, 92429 had published only in one journal, 22585 in two journals, 9996 in three journals, and 16731 in more than three journals. The "*World Journal of Gastroenterology*" had the greatest number of authors (17838), followed by "*Gastroenterology*" (12770), "*Digestive Diseases and Sciences*" (11395), "*American Journal of Gastroenterology*" (10889), and "*Hepatology*" (10588).

CONCLUSION: Global gastroenterology publications displayed a continuous growth in the new millennium. The change was most striking in certain journals. Regular bibliometric analyses on the trends and specific topics would help researchers publish more efficiently and allow editors to adjust the policy more accurately.

Key words: Bibliographic databases; Bibliometrics; Biomedical research; Gastroenterology; MEDLINE

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INTRODUCTION

Gastroenterology is a highly competitive and productive field of medical research. Researchers in this field usually have a stronger need for bibliographic information than those in other fields. In the past few years, several bibliometric researches have been devoted to the global research trend[1], the research output in specific regions[2,3] and countries[4-6], and the share of author origins in specific journals[7-9] in gastroenterology. However, there still seemed to be a lack of a comparative overview of all gastroenterology specialty journals in the world.

The aim of the current study was to analyze the publications in gastroenterology journals in the new millennium, based on MEDLINE which is freely offered over the internet by the National Library of Medicine in the United States of America. Special attention was paid to specific types of articles, e.g. randomized controlled trials. Because researchers might not publish solely in

one journal during a period of several years, a new analytical method was also proposed to calculate the share of authors publishing in only one journal among all authors in each journal. This new indicator may serve as another dimension to author origins in a journal.

MATERIALS AND METHODS

Journal selection and data sources

The specialty journals of gastroenterology were limited to those listed under the subject heading of "gastroenterology" in MEDLINE. The master file of journals in MEDLINE was first downloaded (ftp://ftp.nlm.nih.gov/online/journals/lsi2008.xml, accessed on September 8, 2008) and a total of 141 gastroenterology journals were identified. Due to cessation of publication and name changes, not all journals were still available.

The bibliographic entries of papers belonging to selected journals were downloaded from MEDLINE on the PubMed web site (http://www.ncbi.nlm.nih.gov/sites/entrez/, accessed on September 8, 2008). The downloading consisted of journals with a Perl script. The retrieval was limited to papers published since 2001. The format type of the retrieval was MEDLINE.

Study design

One bibliographic record with the MEDLINE format contains pairs of tags and data, e.g. PMID-14647050 and AU-Lee SD, where PMID (PubMed unique identifier) and AU (author) are tags. Some types of tags, e.g. AU, PT (publication type) and MH (medical subject headings term), might appear more than once in one record. Some types of tags are not obligatory and might not be present in every record.

The downloaded datasets with MEDLINE format were merged and transformed into one single file with the structure of entity-attribute-value (EAV)[10] for further efficient processing, where entity stood for the PMID of a paper, attribute for the tag, and value for the data. For example, the pair "AU-Lee SD" in the paper of "PMID-14647050" would be converted into "14647050 [tab] AU [tab] Lee SD".

From the EAV file, the numbers of papers in each journal during the years of coverage were first calculated. The processing was limited to papers categorized as "journal article" in the publication type field. In addition, only papers published between January 1, 2001 and December 31, 2007 were included in this analysis.

The articles were also counted according to their publication type. An article might not contain only one publication type. For articles with the publication type of randomized controlled trial, their distribution in journals over the years was computed.

In computing the productivity of individual researchers in all gastroenterology journals, the methods of "total author counting"[11] was adopted. Each author of an article was recognized as having written one article, and then the number of articles authored or coauthored by each researcher during the 7-year period was counted.

Because an author's full name has been indexed in MEDLINE since 2002 and one fifth of the original publications did not contain the full author name[12], authors in the current study were identified according to the conventional author indexing of MEDLINE, i.e. last name, up to two initials of first and middle names, and/or a suffix abbreviation. Different authors with the same last name and initials would not be specifically differentiated in aggregate statistics. However, the name ambiguity would be considered in listing the most prolific authors.

For each journal, the total number of authors who had published in the journal during the 7-year period was calculated as the denominator and then the number of authors who had never published in other gastroenterology journals during the 7-year period was computed as the numerator. The fractional number for each journal might suggest the breadth of author origins.

Statistical analysis

The programming scripts with Perl version 5.10.0 (http://www.perl.com/) were written for downloading and computing. As a popular computer language since the internet era, Perl belongs to the open source software and can be freely downloaded and distributed for use. The National Library of Medicine also provides examples of Perl scripts for use of Entrez programming utilities from PubMed.

Only descriptive statistics, frequency in count and percentage, were displayed.

RESULTS

According to MEDLINE, 81 561 articles were published in 91 gastroenterology journals from 2001 to 2007 (Table 1). The number of articles increased from 9447 in 2001 to 13 340 in 2007. The "*World Journal of Gastroenterology*" had the largest number of publications (5684 articles), followed by "*Hepato-Gastroenterology*" (3036) and "*Gastrointestinal Endoscopy*" (3005). Only 12 journals had more than 2000 articles indexed in MEDLINE. By comparing the situations in 2001 and in 2007, the "*World Journal of Gastroenterology*" had the highest growth rate of publications (5.0-fold increase), followed by "*BMC Gastroenterology*" (2.3-fold), "*Revista de Gastroenterologia de Mexico*" (2.2-fold), "*International Journal of Colorectal Disease*" (2.0-fold), and "*Inflammatory Bowel Diseases*" (2.0-fold). The highest absolute growth was also claimed by "*World Journal of Gastroenterology*" (867 more articles), followed by "*Journal of Gastroenterology and Hepatology*" (208), "*Zhonghua Ganzangbing Zazhi* (*Chinese Journal of Hepatology*)" (200), and "*Digestive Diseases and Sciences*" (180). In contrast, 21 journals had fewer publications in 2007 than in 2001.

As to the publication type of these articles, reviews accounted for 17.2% (14 005 articles) of all articles from 2001 to 2007 and case reports 15.4% (12 539) (Table 2). There were only 3739 randomized controlled trials (4.6% of all articles) among 4627 clinical trials (5.7%). The annual number of randomized controlled trials increased from 442 in 2001 to 572 in 2007. Randomized controlled trials were most frequently published in "*Alimentary Pharmacology & Therapeutics*" (536 articles), followed by

Table 1 Publication trend of articles in gastroenterology journals, 2001-2007

Journal	2001	2002	2003	2004	2005	2006	2007	Total
Abdom Imaging	126	117	147	116	119	146	126	897
Acta Gastroenterol Belg	45	52	38	45	66	51	47	344
Acta Gastroenterol Latinoam	34	17	30	16	27	52	49	225
Aliment Pharmacol Ther	246	305	372	416	363	390	338	2430
Am J Gastroenterol	543	466	425	338	363	383	340	2858
Am J Physiol Gastrointest Liver Physiol	331	289	267	272	298	289	354	2100
Ann Hepatol		30	29	29	48	56	47	239
Arq Gastroenterol	44	42	45	49	46	61	63	350
Best Pract Res Clin Gastroenterol	64	67	66	90	66	68	67	488
BMC Gastroenterol	14	23	34	32	39	43	46	231
Can J Gastroenterol	98	79	98	95	66	85	97	618
Chin J Dig Dis				35	43	39		117
Clin Colorectal Cancer	27	31	34	43	60	54	49	298
Clin Gastroenterol Hepatol			62	153	219	231	275	940
Clin Liver Dis	52	56	53	49	44	48	50	352
Colorectal Dis	68	105	93	92	104	139	149	750
Curr Gastroenterol Rep	81	70	79	77	76	71	80	534
Curr Issues Intest Microbiol	5	5	8	7	7	10	5	47
Curr Opin Gastroenterol	91	85	66	78	86	81	78	565
Dig Dis	47	40	43	53	36	33	53	305
Dig Dis Sci	390	415	345	324	406	376	570	2826
Dig Liver Dis	129	172	174	166	153	176	200	1170
Dig Surg	95	96	78	70	60	65	78	542
Digestion	88	69	62	71	69	77	70	506
Dis Colon Rectum	263	245	255	270	301	246	257	1837
Dis Esophagus	63	68	75	66	80	91	92	535
Dysphagia	37	42	36	35	42	40	43	275
Eat Weight Disord	35	42	55	49	56	52	47	336
Eksp Klin Gastroenterol		145	120	94	96	81	119	655
Eur J Gastroenterol Hepatol	263	222	212	217	217	216	192	1539
Gastric Cancer	33	45	57	35	42	45	40	297
Gastroenterol Clin Biol	196	195	185	213	172	190	177	1328
Gastroenterol Clin North Am	54	74	54	59	46	49	51	387
Gastroenterol Hepatol	89	90	87	109	95	142	84	696
Gastroenterol Nurs	48	53	47	55	65	68	51	387
Gastroenterology	361	437	402	468	428	439	456	2991
Gastrointest Endosc	436	491	447	429	374	412	416	3005
Gastrointest Endosc Clin N Am	47	55	52	59	51	61	52	377
Gut	322	406	348	346	342	320	299	2383
Hepatobiliary Pancreat Dis Int		128	121	128	120	115	114	726
Hepatogastroenterology	414	422	580	443	426	209	542	3036
Hepatology	344	389	335	353	319	346	388	2474
Hernia	47	44	50	84	86	104	98	513
Indian J Gastroenterol	104	87	105	85	88	104	93	666
Inflamm Bowel Dis	66	70	65	156	160	147	200	864
Int J Colorectal Dis	63	64	86	89	74	123	191	690
Int J Gastrointest Cancer	43	46	43		51	23		206
Int J Pancreatol	25							25
J Clin Gastroenterol	185	180	156	177	177	177	157	1209
J Dig Dis							36	36
J Gastroenterol	125	206	192	170	156	147	172	1168
J Gastroenterol Hepatol	239	279	213	265	327	365	447	2135
J Gastrointest Surg	100	127	140	161	175	195	258	1156
J Gastrointestin Liver Dis						61	69	130
J Health Popul Nutr	32	45	43	47	44	57	56	324
J Hepatobiliary Pancreat Surg	88	107	76	84	91	97	98	641
J Hepatol	226	231	304	268	282	276	227	1814
J Pediatr Gastroenterol Nutr	256	268	207	234	273	244	219	1701
J Viral Hepat	62	64	71	80	90	117	126	610
JOP	55	18	24	68	69	79	87	400
Korean J Gastroenterol			81	103	136	144	133	597
Korean J Hepatol				41	51	57	59	208
Liver	58	87						145
Liver Int			75	96	153	159	172	655
Liver Transpl	184	186	234	256	225	297	281	1663
Minerva Gastroenterol Dietol	27	42	32	35	29	41	36	242
Nat Clin Pract Gastroenterol Hepatol				27	126	121	138	412
Neurogastroenterol Motil	55	64	64	114	104	105	128	634
Nippon Shokakibyo Gakkai Zasshi	127	155	141	112	130	120	144	929

Pancreas	128	134	154	147	143	116	131	953
Pancreatology	74	58	55	38	72	54	56	407
Rev Esp Enferm Dig	57	61	73	99	89	91	110	580
Rev Gastroenterol Disord	13	28	47	51	35	27	32	233
Rev Gastroenterol Mex	31	83	66	82	105	88	99	554
Rev Gastroenterol Peru	34	32	34	35	39	39	46	259
Rom J Gastroenterol		48	47	50	63			208
Ross Gastroenterol Zh	29							29
Scand J Gastroenterol	218	235	209	222	219	210	235	1548
Scand J Gastroenterol Suppl	16	18	30	15		25		104
Semin Gastrointest Dis	26	23	23					72
Semin Liver Dis	43	41	44	60	47	39	38	312
Surg Endosc	376	454	505	393	292	362	463	2845
Surg Laparosc Endosc Percutan Tech	86	89	90	80	92	107	144	688
Taehan Kan Hakhoe Chi		67	42					109
Tech Coloproctol	41	43	39	130	54	67	59	433
Trop Gastroenterol	65	66	59	55	56	43	51	395
Turk J Gastroenterol		48	59	58	53	65	49	332
World J Gastroenterol	172	236	632	812	1478	1315	1039	5684
Z Gastroenterol	134	156	127	115	90	90	93	805
Zhonghua Ganzangbing Zazhi	114	193	325	325	341	308	314	1920
Zhonghua Weichang Waike Zazhi					109	108	105	322
Total	9447	10663	11178	11663	12610	12660	13340	81561

"*American Journal of Gastroenterology*" (278), "*Gastrointestinal Endoscopy*" (186), "*Surgical Endoscopy*" (183), "*World Journal of Gastroenterology*" (176), and "*Gastroenterology*" (171). Among these journals, the "*World Journal of Gastroenterology*" had the greatest increase in randomized controlled trials: from 5 in 2001 to 34 in 2007 (detailed data not shown in tables).

If only the last name and initials of the authors were considered, 141 741 author names appeared in the articles of gastroenterology journals from 2001 to 2007. The "*World Journal of Gastroenterology*" had the greatest number of authors (17 838), followed by "*Gastroenterology*" (12 770), "*Digestive Diseases and Sciences*" (11 395), "*American Journal of Gastroenterology*" (10 889), and "*Hepatology*" (10 588) (Table 2). Among all authors, 82 174 had published only one article, 22 192 two articles, 10 672 three articles, and 26 703 more than three articles. On the other hand, 92 429 authors had published only in one journal, 22 585 in two journals, 9996 in three journals, and 16 731 in more than three journals. The share of authors publishing only in one journal among all authors of the journal was generally higher in journals with apparently narrower research fields or locality, e.g. "*Eksperimental'naia i Klinicheskaia Gastroenterologiia* (*Experimental & Clinical Gastroenterology*)" (95.5%), "*Revista de Gastroenterologia del Peru*" (84.0%), "*Eating and Weight Disorders*" (81.0%), "*Revista de Gastroenterologia de Mexico*" (79.4%), and "*Journal of Health, Population, and Nutrition*" (78.0%) (Table 2).

The top 10 prolific researchers in these gastroenterology journals are listed in Table 3. They were from seven institutions in six countries: three in the USA, four in Europe, and three in Asia. Only two of the top-ranked researchers were surgeons (Masatoshi Makuuchi and Markus W Buchler).

DISCUSSION

The current study demonstrated the most recent trend in publications from gastroenterology specialty journals worldwide. Gastroenterology publications have continued to prosper in the new millennium, not merely due to the expanded coverage of MEDLINE or the growth of a single journal. The number of randomized controlled trials has also increased, but their growth rate has slightly lagged behind that of other articles. Numerous researchers participated in gastroenterology publications; a substantial number of the authors were active in research and had multiple publications. Gastroenterology journals thus showed diverse authorships in which many authors of a journal also published in other specialty journals.

The current study chose MEDLINE as the data source because of its open access and international visibility. To compare the "quality" of scientific publications, people have adopted the controversial citation statistics and "impact factor" in recent years. Because the quantity of citations has increased tremendously and the databank of citations is not freely open to the public, the monopolized data from the black box cannot be extensively verified. Normally, most researchers just need a quick orientation in the field of interest, e.g. the features of journals, the most prolific authors or facilities, or the hottest subjects. Such requests can be easily satisfied by free MEDLINE after processing, without resorting to commercial databases which most individual researchers around the world can hardly afford.

Despite collective growth since 2001, the increases and decreases in individual gastroenterology journals could be observed. Among all journals, the "*World Journal of Gastroenterology*" was most striking. Not only had it published the greatest number of articles since 2003, but it had also attracted the most authors. Along with quantitative growth, the "*World Journal of Gastroenterology*" also had more randomized controlled trials. According to an earlier bibliometric analysis on the "*World Journal of Gastroenterology*", the author origins of the Journal had become more diverse by geographic distribution since 2003. From the analysis in the current study, the majority

Table 2 Articles of gastroenterology journals stratified by selected publication type and author type, 2001-2007

Journal	No. of all articles	Review	Case report	Clinical trial	Multicenter study	Randomized controlled trial	No. of authors	No. of exclusive authors[1]	Share of exclusive authors[1] (%)
Abdom Imaging	897	184	310	7	1	7	3094	1360	44.0
Acta Gastroenterol Belg	344	137	92	8	4	3	1113	435	39.1
Acta Gastroenterol Latinoam	225	36	52	5	4	2	815	542	66.5
Aliment Pharmacol Ther	2430	674	10	556	273	536	8177	2108	25.8
Am J Gastroenterol	2858	302	169	279	150	278	10889	2753	25.3
Am J Physiol Gastrointest Liver Physiol	2100	126		55		27	7045	2689	38.2
Ann Hepatol	239	89	58	10	2	7	705	332	47.1
Arq Gastroenterol	350	34	21	6	5	10	1067	689	64.6
Best Pract Res Clin Gastroenterol	488	459	6				1145	341	29.8
BMC Gastroenterol	231	6	47	12	6	13	934	164	17.6
Can J Gastroenterol	618	187	137	17	18	18	1579	556	35.2
Chin J Dig Dis	117	23	2	2		5	400	71	17.8
Clin Colorectal Cancer	298	130	25	25	17	14	910	552	60.7
Clin Gastroenterol Hepatol	940	153	198	64	76	89	4079	918	22.5
Clin Liver Dis	352	349	1				522	78	14.9
Colorectal Dis	750	91	11	32	14	33	2202	895	40.6
Curr Gastroenterol Rep	534	418	1	6	1	5	791	139	17.6
Curr Issues Intest Microbiol	47	24					133	81	60.9
Curr Opin Gastroenterol	565	245					735	144	19.6
Dig Dis	305	202	5	7	2	8	852	128	15.0
Dig Dis Sci	2826	183	649	148	58	135	11395	3418	30.0
Dig Liver Dis	1170	239	146	78	45	56	4634	1270	27.4
Dig Surg	542	96	148	9	3	13	1997	514	25.7
Digestion	506	106	45	49	28	41	2332	483	20.7
Dis Colon Rectum	1837	130	256	219	57	143	6506	2349	36.1
Dis Esophagus	535	71	145	21	7	15	2226	709	31.9
Dysphagia	275	16	33	13	1	6	850	579	68.1
Eat Weight Disord	336	35	21	17	5	14	1070	867	81.0
Eksp Klin Gastroenterol	655	138	23	76	1	18	984	940	95.5
Eur J Gastroenterol Hepatol	1539	315	378	83	93	87	6606	1966	29.8
Gastric Cancer	297	40	54	30	9	7	1301	249	19.1
Gastroenterol Clin Biol	1328	603	326	35	36	16	3188	1623	50.9
Gastroenterol Clin North Am	387	373	3				614	107	17.4
Gastroenterol Hepatol	696	229	184	12	3	3	2259	1235	54.7
Gastroenterol Nurs	387	114	29	12	1	9	430	293	68.1
Gastroenterology	2991	352	205	169	106	171	12770	4003	31.3
Gastrointest Endosc	3005	220	1275	230	100	186	8547	2426	28.4
Gastrointest Endosc Clin N Am	377	338	2				581	87	15.0
Gut	2383	343	227	132	139	144	9785	2612	26.7
Hepatobiliary Pancreat Dis Int	726	64	67	40	9	20	2128	464	21.8
Hepatogastroenterology	3036	145	654	155	31	100	9648	2952	30.6
Hepatology	2474	211	15	148	105	131	10588	3502	33.1
Hernia	513	60	151	20	18	32	1627	822	50.5
Indian J Gastroenterol	666	66	355	16	2	17	1791	893	49.9
Inflamm Bowel Dis	864	158	37	43	34	31	3266	854	26.1
Int J Colorectal Dis	690	63	74	57	21	40	3249	930	28.6
Int J Gastrointest Cancer	206	36	52	14	2		968	279	28.8
Int J Pancreatol	25	3	9				137	9	6.6
J Clin Gastroenterol	1209	320	241	59	31	56	4326	1192	27.6
J Dig Dis	36	6	3	2	1	1	160	29	18.1
J Gastroenterol	1168	219	299	45	24	29	4429	734	16.6
J Gastroenterol Hepatol	2135	274	351	93	37	91	7661	1672	21.8
J Gastrointest Surg	1156	114	132	23	16	24	4329	1476	34.1
J Gastrointestin Liver Dis	130	22	41	4	3	3	484	216	44.6
J Health Popul Nutr	324	16	1	8	2	16	1185	924	78.0
J Hepatobiliary Pancreat Surg	641	142	207	8	4	6	2345	427	18.2
J Hepatol	1814	259	114	120	56	81	8036	2200	27.4
J Pediatr Gastroenterol Nutr	1701	257	389	93	36	119	5894	3239	55.0
J Viral Hepat	610	74	16	81	48	56	3255	887	27.3
JOP	400	124	168	7		2	1396	473	33.9
Korean J Gastroenterol	597	65	128	7	4	8	1249	372	29.8
Korean J Hepatol	208	25	28	2		3	585	89	15.2
Liver	145	14	20	14	1	5	759	164	21.6
Liver Int	655	68	38	39	16	19	3613	751	20.8
Liver Transpl	1663	223	261	72	43	49	5517	2025	36.7
Minerva Gastroenterol Dietol	242	87	15	5	1	2	788	284	36.0
Nat Clin Pract Gastroenterol Hepatol	412	128	35	8	10	8	693	91	13.1

Neurogastroenterol Motil	634	111	5	52	5	39	1874	478	25.5
Nippon Shokakibyo Gakkai Zasshi	929	326	594	1	1	1	3464	856	24.7
Pancreas	953	60	109	35	16	9	3966	1144	28.8
Pancreatology	407	108	55	16	5	2	1627	399	24.5
Rev Esp Enferm Dig	580	80	145	28	5	13	2185	1234	56.5
Rev Gastroenterol Disord	233	154	4				124	10	8.1
Rev Gastroenterol Mex	554	204	112	15		8	1224	972	79.4
Rev Gastroenterol Peru	259	50	74	8		6	824	692	84.0
Rom J Gastroenterol	208	51	66	24	5	4	590	318	53.9
Ross Gastroenterol Zh	29	5	2	2			90	44	48.9
Scand J Gastroenterol	1548	80	128	151	65	120	6465	1817	28.1
Scand J Gastroenterol Suppl	104	92	2	1	1		270	28	10.4
Semin Gastrointest Dis	72	64	43				121	11	9.1
Semin Liver Dis	312	264	29	2			598	80	13.4
Surg Endosc	2845	229	430	203	73	183	9117	4404	48.3
Surg Laparosc Endosc Percutan Tech	688	45	318	23	4	24	2523	871	34.5
Taehan Kan Hakhoe Chi	109	4	30	4		2	417	61	14.6
Tech Coloproctol	433	53	82	26	14	23	1283	500	39.0
Trop Gastroenterol	395	84	166	11	1	7	929	440	47.4
Turk J Gastroenterol	332	11	87	7	4	6	1096	460	42.0
World J Gastroenterol	5684	584	637	338	46	176	17838	6839	38.3
Z Gastroenterol	805	198	193	39	14	14	1917	737	38.4
Zhonghua Ganzangbing Zazhi	1920	95	33	34	11	34	3693	1117	30.2
Zhonghua Weichang Waike Zazhi	322						1096	262	23.9
Total	81561	14005	12539	4627	2090	3739	141741	92429	65.2

[1]Exclusive authors denote the authors who had published in only one journal during the study period.

Table 3 The most prolific authors in gastroenterology journals, 2001-2007

Author	Affiliation	No. of articles
Nicholas J Talley	Mayo Clinic, Rochester, USA	205
Michael P Manns	Hannover Medical School, Germany	170
Peter Malfertheiner	University of Magdeburg, Germany	166
Todd H Baron	Mayo Clinic, Rochester, USA	165
Masatoshi Makuuchi	University of Tokyo, Japan	163
Markus W Buchler	University of Heidelberg, Germany	160
Shou-Dong Lee	Taipei Veterans General Hospital, Taiwan, China	155
Giovanni Gasbarrini	Catholic University of Rome, Italy	147
William J Sandborn	Mayo Clinic, Rochester, USA	141
Full-Young Chang	Taipei Veterans General Hospital, Taiwan, China	140

of authors in the "*World Journal of Gastroenterology*" did not only publish in this Journal during the study period. Both of these facts indicated that the "*World Journal of Gastroenterology*" had established its position in international gastroenterology publications.

The global research community has frequent fervent disputes about the quality of journal articles[13]. There appears to be a growing discontent about the misuse of the impact factor in hiring, promoting and grant-awarding. Researchers usually consider several factors when choosing a journal to publish their research results. A research article should be judged by its content, and not merely by the journal in which it was published. That is, the impact of an individual article should not be evaluated by the journal impact factor. The function of the academic journal as an effective platform for scientific communication can never be overestimated. Time will show which editorial team acts best.

The major limitation in the current study was the separation of distinct authors. The ambiguity of author names is an unresolved problem of bibliometric research[14]. Although MEDLINE started to index full author names in 2002, the problem still remains. Not every journal print author names in full. Besides, not every author spells their forename consistently in different articles. For example, Buchler MW appeared as Markus W Buchler, Markus-W Buchler, and Markus Wolfgang Buchler. Most cases of ambiguity existed in authors of East Asian origin. Because of homonymous features in Chinese/Japanese/Korean characters, a lot of distinct authors might share the same full name spelled in Latin letters. Therefore, the share of exclusive authors (never publishing in other journals) in each journal in the current study represented only the lowest estimate.

In conclusion, global gastroenterology publications demonstrated a continuous growth in the new millennium. The change was most striking in certain journals. Regular bibliometric analyses on the trends and specific topics would help researchers publish more efficiently and allow editors to adjust the policy more accurately.

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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
London, UK
Gastro 2009 UEGW/World Congress of Gastroenterology
www.gastro2009.org

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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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Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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Write as mean ± SD or mean ± SE.

Statistical expression

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EDITORIAL

Acute pancreatitis at the beginning of the 21st century: The state of the art

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Abstract

Acute pancreatitis is an acute inflammatory disease of the pancreas which can lead to a systemic inflammatory response syndrome with significant morbidity and mortality in 20% of patients. Gallstones and alcohol consumption are the most frequent causes of pancreatitis in adults. The treatment of mild acute pancreatitis is conservative and supportive; however severe episodes characterized by necrosis of the pancreatic tissue may require surgical intervention. Advanced understanding of the pathology, and increased interest in assessment of disease severity are the cornerstones of future management strategies of this complex and heterogeneous disease in the 21st century.

Key words: Acute necrotizing pancreatitis; Systemic inflammatory response syndrome; Surgery; Pancreatectomy; Minimal surgical procedures

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INTRODUCTION

The death of Alexander the Great (356-323 BC) at the age of 33 has been ascribed to acute necrotizing pancreatitis secondary to rich food and heavy alcohol consumption[1].

In 1856, the great French physiologist Claude Bernard (1813-1878) demonstrated the capacity of pancreatic secretions to digest proteins, carbohydrate and fat[2]. However, the first review on acute pancreatitis (AP) was published by Reginald Huber Fitz (1843-1913) in 1889[3]. In his observation of 53 patients with clinical signs of AP, he believed that the disease was a complication of gastroduodenitis causing inflammation of the biliary duct.

Nevertheless, only in late 19th century did Chiari propose pancreatic autodigestion as the main pathological mechanism of the disease[4]. This theory facilitated the understanding of the late 19th and early 20th century clinicians in the pathophysiology of AP. In 1901, Eugen Opie proposed the "common channel hypothesis", which was based on the assumption that a gallstone lodged in the ampulla could occlude both the common bile duct (CBD) and the pancreatic duct. The obstruction caused the formation of a common channel that would allow reflux of bile into the pancreatic duct with activation of pancreatic enzymes and pancreatitis[5,6].

The pathophysiology and treatment of AP has been intensely studied during the last century and our aim is to review recent evidence and achievements in the diagnosis and treatment of this serious condition at the beginning of the 21st century.

EPIDEMIOLOGY

AP is a growing problem in Europe, posing significant medical, surgical and financial sequelae[7]. A recent systematic review presented trends in incidence of the first attack of AP using data from 12 longitudinal studies[8]. The mean age of the first attack was in the 6th decade. This outcome can be explained by an increasing

incidence of gallstone pancreatitis among white women over the age of 60 years[9,10]. The most common causes were gallstones (11%-56%), idiopathic (8%-44%) and alcohol (3%-66%). However, occult microlithiasis is probably responsible for most cases of idiopathic AP[11].

Gallstone AP was found to be more common in female subjects, alcohol pancreatitis more common amongst men and idiopathic pancreatitis similar in both sexes.

The incidence of AP has been reported to be markedly increasing[8,12,13]. The explanation of this increased incidence could be explained by the routine testing of pancreatic enzymes in patients presenting with abdominal pain at emergency departments, and in over-diagnosis in cases of non-specific increases in enzymes due to other causes. Another explanation is an increase in the incidence of gallstone disease and obesity in the population.

Although fatalities associated with AP have decreased over time from 15%-20% to below 5%, the population mortality rate has remained unchanged with increasing age associated with higher mortality[14-16]. A correlation with duration of disease was also shown with 65% of the deaths occurring in the first 14 d and 80% within 30 d[17].

Overall, severe AP (SAP) occurs in 10%-20% of patients and despite improvements in critical care between 10% and 25% of patients with SAP die[18,19].

PATHOPHYSIOLOGY

The pathogenesis of AP is caused by an inappropriate activation of trypsinogen to trypsin. Once activated these enzymes are responsible of autodigestion of pancreatic tissues resulting in necrosis of the acini and pancreatic islets with interstitial fat necrosis and necrotizing vasculitis[20].

These pathological changes in the pancreatic gland are responsible for releasing active pancreatic enzymes into the bloodstream and stimulating the production of inflammatory cytokines such as interleukin-1, interleukin-6 and interleukin-8 from neutrophils, macrophages and lymphocytes. The release of those interleukins and tumor necrosis factor-α (TNF-α) from macrophages triggers an inflammatory cascade which leads to the systemic inflammatory response syndrome (SIRS)[21]. SIRS may develop into acute respiratory distress syndrome or multiorgan dysfunction syndrome. This systemic inflammatory response to pancreatic injury marks the "first or early phase" of the natural course of SAP, which normally characterizes the first 14 d of the disease[21,22]. In this phase, organ failure is common and often is not associated with infection[23]. The "second or late phase" which starts 14 d after the onset of the disease, is marked by infection of the gland, necrosis and septic systemic complications causing a significant increase in mortality[24]. Infection of the necrotic pancreas occurs in the 8% to 12% of the patients with AP and in 30% to 40% of patients with necrotizing pancreatitis, and it is considered the most important risk factor of necrotic pancreatitis[25,26].

DIAGNOSIS

Abdominal pain together with elevation of plasma levels of pancreatic enzymes is the cornerstone of diagnosis. The pain normally is generalized in the upper abdomen and occurs suddenly without a prodrome. The pain, which tends to last a few days, is often radiated in a bandlike manner to the lower thoracic region of the back. Nausea and vomiting normally appear in about 90% of patients and can be severe. Physical signs of severe disease such as ecchymoses in the flank (Gray-Turner's sign) or in the periumbilical region (Cullen sign) occurs in less than 3% of patients, and have been associated with a mortality of 37%[27]. Pancreatic enzymes are released into the circulation during an acute attack. Levels peak early, and decline over 3-4 d. As a consequence, the diagnosis should not rely on arbitrary levels 3 or 4 times greater than normal, but levels should be interpreted in light of the time since the onset of abdominal pain.

Enzymes released by acinar cells during an attack of AP are amylase and lipase, and their concentration in the serum is used to confirm the diagnosis[28]. The half-life of elevated amylase is shorter than that of lipase: the diagnosis using plasma lipase has slightly superior sensitivity and specificity and greater overall accuracy than amylase.

There are more specific tests in detection of AP, such as urinary trypsinogen activation peptide (TAP), serum and urinary trypsinogen[29-32], but these are less widely available.

Other laboratory tests can exclude metabolic causes such as hypercalcemia and lipid disorders.

AETIOLOGY

In order to optimize the instant management and prevent recurrence of AP it is essential identify the aetiology.

In the Western world, biliary tract disease (38%) and alcoholism (36%) are accountable for the majority of cases of AP[33]. However, in up to 10% of cases, the cause of AP remains unknown (idiopathic AP).

Gallstones

In patients with no history of alcohol consumption, an increased level of serum alanine aminotransferase up to 3 times its normal value is indicative of gallstone pancreatitis.

Gallstone pancreatitis, in most cases, is caused by gallstones passing into the bile duct and temporarily lodging at the sphincter of Oddi. However, duct obstruction can also be localized in the pancreatic duct. Although not completely proven, it is thought that duct obstruction leads to increased pancreatic duct pressure with subsequence injury to acinar cells and activation of digestive enzymes. It is supposed that only gallstones with a diameter up to 5 mm can migrate distally into the biliary duct whilst gallstones with a diameter of 8 mm or more remain in the gallbladder[34].

However, a biliary aetiology should not be excluded when liver function tests are normal since 15%-20% of patients with biliary AP can have normal concentrations of hepatic enzymes[35].

In cases where a biliary aetiology is suspected, the first line of investigation should be a trans-abdominal ultrasound (T-A US). The value of US lies in its ability to demonstrate gallbladder stones and dilatation of the CBD as well as other pathology unrelated to the pancreas. In the case of high clinical suspicion of a biliary cause of AP with normal T-A US, magnetic resonance cholangiopancreatography or endoscopic US should be performed in order to visualize the presence of microlithiasis or other causes of duct obstruction.

Alcohol

Alcohol consumption is the second cause of AP. Although the acinar cell is considered the main target of damage by ethanol, there is not an accepted explanation for why some patients are more predisposed to developing AP than others who consume similar quantities of alcohol. The pathogenesis of alcohol pancreatitis can be explained by a combination of environmental and genetic factors. Genetic studies have suggested that, in hereditary pancreatitis, mutation of the cationic trypsinogen gene and serine peptidase inhibitor, Kazal type 1 (SPINK1) genes can promote AP in the presence of alcohol[36].

Post-ERCP acute pancreatitis

The risk of developing AP after endoscopic retrograde cholangiopancreatography (ERCP) is around 5%[37]. The main risk factors for post-ERCP AP include female gender, presence of periampullary diverticulum, and procedure-related factors such as a cannulation time of more than 10 min and major papilla sphincterotomy. However, the risk of developing asymptomatic hyperamylasemia, which appears in 35%-70% of patients, seems to be linked with procedure-related factors[38].

Trauma

Abdominal trauma causes an elevation of amylase and lipase levels in 17% of cases and clinical AP in 5% of cases. Pancreatic injury occurs more often in penetrating injuries (e.g. from knives, bullets) than in blunt abdominal trauma (e.g. from steering wheels, horses, bicycles). Blunt injury may crush the gland across the spine, leading to a ductal injury in that location[39].

Drug-induced pancreatitis

Drug-induced pancreatitis is considered a rare event (0.1%-2%) and is normally mild and self-limiting. In the literature, the true incidence of drug-induced AP is not known since the evidence is derived mainly from case reports and the diagnosis is always challenging because of the difficulty in distinguishing the effects of drugs from other causes of AP[40].

Drugs strongly associated with AP include azathioprine, sulfonamides, sulindac, tetracycline, valproic acid, didanosine, methyldopa, estrogens, furosemide, 6-mercaptopurine, pentamidine, 5-aminosalicylic acid compounds, corticosteroids, and octreotide.

Infections

Infections are accountable for less than 1% of all AP and tend to be milder than biliary and alcohol-induced AP[41]. Viral infections such as Epstein-Barr, coxsackie virus, echovirus, varicella-zoster and measles are the most common causes of infectious AP especially in children.

Bacterial causes include *Mycoplasma pneumoniae*, *Salmonella typhosa*, *Leptospira*, *Campylobacter* and *Mycobacterium tuberculosis*.

Worldwide, ascariasis can cause AP by migration of worms in and out of the duodenal papillae.

Hereditary pancreatitis

Hereditary pancreatitis is an autosomal dominant gain-of-function disorder related to mutations of the cationic trypsinogen gene (*PRSS1*), which has an 80% penetrance. Mutations in this gene cause premature conversion of trypsinogen to active trypsin causing pancreatic autodigestion. This genetic syndrome is associated with a high risk of developing chronic pancreatitis at a young age and of developing pancreatic cancer[42].

Mutations in the SPINK1 gene, which blocks the active binding site of trypsin, rendering it inactive, are associated with acute and chronic pancreatitis. Patients who have severe SPINK1 mutations normally develop chronic pancreatitis in childhood[43].

In patients with mild CFTR gene mutations, an increased risk of developing acute and chronic pancreatitis has been observed in comparison with the general population.

Hypercalcemia

Hypercalcemia and primary hyperparathyroidism can lead to AP. Hypercalcemia, which causes less than 1% of all cases of pancreatitis, normally appears with excessive doses of vitamin D, familial hypocalciuric hypercalcemia and total parenteral nutrition.

Hypertriglyceridemia

AP usually does not occur until serum triglyceride levels reach 1000 mg/dL. Hypertriglyceridemia causes about 2% of AP and it is normally associated with type I , Type Ⅱ and Type V hyperlipidemia. The triglyceride level should be measured as soon as clinical presentation of AP appears since this level tends to decline during hospitalization because of fasting and Ⅳ fluid resuscitation.

Acquired hypertriglyceridemia can appear in adults because of alcoholism, obesity and poorly controlled diabetes mellitus.

In order to prevent recurrent attacks of AP, the patient should be placed on a low-fat diet, a regular exercise regimen, and tight control of diabetes, with use of lipid-lowering drugs such as statins.

Developmental abnormalities of the pancreas

The pancreas develops from two buds stemming from the alimentary tract of the developing embryo.

Pancreas divisum is a failure of the dorsal and ventral pancreatic ducts to fuse during embryogenesis and it occurs in about 5%-7% of the healthy population. Pancreatitis appears only in 5% of patients with pancreas divisum and it is thought to be the result of ductal hypertension caused

Table 1 Ranson's criteria for prediction of severity of acute pancreatitis

On admission	During initial 48 h
Age > 55 yr	Hemoglobin falls below 10 mg/dL
White cell count < 16×10^9/L	Blood urea nitrogen increases by > 5 mg/dL
Lactate dehydrogenase > 350 U/L	Calcium < 8 mg/dL
Aspartate aminotransferase > 250 U/L	PaO_2 < 60 mmHg (8 kPa)
Glucose > 200 mg/dL	Base deficit > 4 mEq/L
	Fluid sequestration > 6 L

PaO_2: Arterial partial pressure of oxygen.

Table 2 Glasgow (Imrie) severity scoring system for acute pancreatitis

Age > 55 yr
White cell count > 15×10^9/L
PaO_2 < 60 mmHg (8 kPa)
Serum lactate dehydrogenase > 600 U/L
Serum aspartate aminotransferase > 200 U/L
Serum albumin < 32 g/L
Serum calcium < 2 mmol/L
Serum glucose > 10 mmol/L
Serum urea > 16 mmol/L

by a narrow duct at its papillary origin.

Sphincter of Oddi dysfunction (SOD) is suspected clinically by recurrent episodes of epigastric or right upper quadrant pain that last 30 min or longer and that are not relieved by bowel movements or by antiacids[44]. SOD can lead to AP by causing increased pancreatic ductal pressure. However, SOD is a controversial cause of AP especially in patients without elevated sphincter pressures on manometry.

Tumor

Obstruction of the pancreatic ductal system by a tumor can increase the intraduct pressure and causing AP in proximately 14% of patients suffering from pancreatic tumors.

Pancreatic ductal carcinoma, ampullary carcinoma, islet cell tumor, solid pseudotumor of the pancreas, sarcoma, lymphoma, cholangiocarcinoma, or metastatic tumor can cause AP.

In addition, a pancreatic cystic neoplasm, such as intraductal papillary-mucinous neoplasm (IPMN), mucinous cystadenoma, or serous cystadenoma, can also cause AP[45].

Postoperative

AP may occur in the postoperative period of various surgical procedures. The mechanism of this pancreatitis includes transient intraoperative hypotension or pancreatic trauma by intraoperative manipulation. Postoperative AP is often a difficult diagnosis to confirm, and it has a higher complication rate than pancreatitis associated with other etiologies.

Autoimmune pancreatitis

This relatively newly described entity is an extremely rare cause of AP. The diagnosis of autoimmune pancreatitis has to be confirmed by specific radiological and histological findings. Radiologically, there is a focal mass in the pancreatic head on computed tomography (CT) scan and irregular narrowing of the proximal pancreatic duct on ERCP.

Patients normally have an elevated Ig G4 level in the serum and infiltration of IgG4-containing plasma cells in the pancreas. It normally occurs in young people who also suffer from inflammatory bowel disease, primary sclerosing cholangitis, primary biliary cirrhosis and Sjogren's syndrome. The therapy of choice is based on steroids.

PREDICTION OF SEVERITY

Although, the majority of patients have a mild episode of AP, it is difficult to identify the patients who are at risk of developing severe disease on admission to the hospital.

There is agreement that there is still a need for an early objective measure of severity. Clinical examination in the first 24 h of admission although specific, lacks sensitivity and hence is unreliable and should be supported by objective measures[46].

Although there is no ideal single serum marker for predicting severity, C-reactive protein (cut-off of 150 mg/L) is a useful indicator of necrosis with a sensitivity and specificity of 80% but is required to be measured more than 48 h after the onset of symptoms[47-49].

Different markers of severity shown to be useful on admission are serum procalcitonin and urinary TAP and trypsinogen-2[50], serum interleukins-6 and -8 and polymorphonuclear elastase at 24 h after admission[51].

The severity of the inflammatory response to pancreatic injury and the presence of organ failure are normally assessed by scoring systems. Multi-factorial scoring systems based on clinical and laboratory findings such as the Ranson's score, Glasgow Score and the Acute Physiology and Chronic Health Evaluation Ⅱ (APACHE Ⅱ) are considered accurate predictors of the severity of AP[52,53].

In 1974, John Ranson selected 11 prognostic signs based on statistical analysis of 43 parameters gathered retrospectively from 450 AP patients. Five of these criteria are measured on admission and the remaining 11 are measured 48 h post admission (Table 1).

The Glasgow severity scoring system considered 9 variables and can be applied from admission although it is not complete for 48 h[54] (Table 2).

Developed in 1985, the APACHE Ⅱ score has been used to predict severity in AP patients[55]. APACHE Ⅱ measures 12 physiological variables and additional points are added based on patient age and on severe baseline chronic diseases. Measuring APACHE Ⅱ score daily can allow an assessment of progression of the disease[56].

Other markers should also be considered such as body mass index (BMI) since obesity has been shown to increase

Table 3 Modified multiple organ dysfunction score

Organ system involved	Score				
	1	2	3	4	5
Cardiovascular PAHR (beats/min)	⩽ 10	10-15	30-15	20-30	> 30
Respiratory PaO_2/FiO_2 (mmHg)	> 300	300-225	150-225	75-150	< 75
Renal creatinine (μmol/L)	< 100	100-200	200-350	350-500	> 500
Neurological glasgow coma score	15	14-13	12-10	9-7	⩽ 6
Hematological platelet count ($\times 10^9$/L)	> 120	80-120	50-80	20-50	⩽ 20
Hepatic bilirubin (μmol/L)	< 20	20-60	60-120	120-240	> 240

PAHR: Pressure-adjusted heart rate [heart rate × (right atrial pressure/mean arterial pressure]; FiO_2: Fraction of inspired oxygen.

the risk of systemic complications and mortality[57]. This has led to a modification of the APACHE II scoring system which includes up to 2 points for obesity[58]. This system is called APACHE-O and although Johnson and colleagues have reported its superiority in predicting outcomes, other authors have not confirmed these results[59].

Although these scoring systems can help the physician in a first assessment of the patient, the most important distinction in terms of prediction of severity is the presence of severe manifestations of the disease such as evidence of SIRS and presence of organ failure.

Mofidi, in a recent retrospective study of 259 patients admitted with AP, showed that the mortality rate was significantly higher in patients who developed or had persistent SIRS at 48 h after admission (25.4%) than in patients who had transient SIRS (8%) or no SIRS in the first 48 h (0.7%)[60].

Therefore, immediate assessment should include clinical evaluation particularly of any cardiovascular, respiratory and renal compromise, BMI, chest X-ray and different acute diseases scores. The presence of any single and/or multiple organ failure has been increasingly recognized as an important variable for predicting mortality from AP. The most common organ dysfunction scores used for critically ill patients are the Multiple Organ Dysfunction Score (MODS) and the Sequential Organ Failure Assessment (SOFA)[61,62] (Tables 3 and 4).

Since the SOFA score uses the mean arterial pressure and therapeutic interventions with vasopressors, its outcome prediction for cardiovascular dysfunction is considered better than the MODS[63].

Since the mortality in the presence of pancreatic necrosis increases from 1% to 10%-23%, the importance of early detection of pancreatic necrosis is not to be overlooked[64]. Contrast-enhanced CT has been considered the "gold standard" for the diagnosis of pancreatic necrosis[65,66].

However, it is not clear how soon the full extent of the necrotic process occurs, but it is at least 4 d after onset of the symptoms, and early CT may therefore underestimate the final severity of the disease. Finally, unless some management decision is required based on the extent of necrosis (for example use of prophylactic antibiotics), CT for staging is unlikely to materially affect the management of patients with AP during the first week of the illness. If CT staging of AP is required, the CT severity index (CTSI) as proposed by Balthazar should be used (Table 5). Recent studies demonstrated that AP patients with a CTSI higher than 5 had 8 times higher mortality. Moreover, they were 17 times more likely to have a prolonged hospital course and were 10 times more likely to require necrosectomy than their counterparts with CT scores < 5[67]. There is also evidence that the site of pancreatic necrosis is an important prognostic factor with a worse outcome observed in patients with necrosis affecting the head of the pancreas[68]. The finding of free intraperitoneal fluid and extensive peri-pancreatic fat stranding have also been demonstrated to be associated with worse outcomes.

Although CT is useful in detecting pancreatic necrosis, it is not able to detect a super-infection of necrosis in the later stage of the disease unless gas bubbles are seen within the necrotic area[69].

Patients with persisting organ failure, or in whom new organ failure develops, and in those with persisting pain and signs of sepsis, will require evaluation by dynamic contrast enhanced CT. CT evidence of necrosis correlates well with the risk of other local and systemic complications.

Since pancreatic necrosis commonly remains stable in appearance, a follow-up CT scan at 3 to 4 wk is not normally considered[70].

MANAGEMENT OF MILD AP

In most cases AP is mild and its initial management is directed towards maintenance of adequate organ perfusion in order to reduce the systemic complications caused by the pancreatic injury. This consists of fluid resuscitation, analgesia, oxygen administration, antiemetics and repeated evaluation of the patient's vital signs with the intention of identifying early manifestations of organ dysfunction.

Since fluid loss in mild pancreatitis can be significant, appropriate fluid resuscitation is a crucial part of the management in order to improve the microcirculation of the pancreas (Figure 1).

As a marker of third-space losses, hemoconcentration has been associated with a higher probability of developing pancreatic necrosis and organ failure[71]. Moreover, patients who experienced a worsening or a lack of improvement of their hemoconcentration after 24 h of fluid resuscitation have a higher chance of developing necrotizing pancreatitis[72]. Therefore, all patients with AP should receive very close clinical monitoring with emphasis on vigorous fluid resuscitation. Rehydratation has to be monitored with the help of invasive monitoring such as a Foley catheter and a central line to measure the urine output and central venous pressure, respectively. Patients who have borderline cardiac dysfunction or respiratory failure may require a Swann-Ganz catheter. Crystalloids are preferred to colloids in most instances. However, electrolyte disturbances and fluid overload can

Table 4 Sequential organ failure assessment score (SOFA)

Organ system involved	Score				
	1	2	3	4	5
Cardiovascular	No hypotension	MAP < 70 mmHg	Dopamine or dobutamine (any dose)	Dopamine > 5 μg/kg per min or adrenaline (epinephrine) < 0.1 μg/kg per min or noradrenaline (norepinephrine) < 0.1 μg/kg per min	Dopamine < 0.1 μg/kg per min or > 15 μg/kg per min or adrenaline > 0.1 μg/kg per min or noradrenaline > 0.1 μg/kg per min
Respiratory PaO_2/FiO_2 (mmHg)	> 400	400-300	300-200	200-100[1]	≤ 100[1]
Renal creatinine (μmol/L)	< 100	100-200	200-350	350-500	> 500
Neurological glasgow coma score	15	14-13	12-10	9-7	≤ 6
Haematological platelet count ($\times 10^9$/L)	> 150	150-100	100-50	20-50	≤ 20
Hepatic bilirubin (μmol/L)	< 20	20-60	60-120	120-240	> 240

[1]These values are calculated with ventilatory support. MAP: Mean arterial pressure; SOFA: Sequential organ failure assessment, and is calculated as the sum of the scores for the individual organs.

Table 5 Acute pancreatitis graded with CT and CT severity index table

Grade	CT finding	Points	Necrosis		Severity index
			Percentage	Additional points	
A	Normal pancreas	0	0	0	0
B	Pancreatic enlargement	1	0	0	1
C	Pancreatic inflammation and/or peripancreatic fat	2	< 30	2	4
D	Single peripancreatic fluid collection	3	30-50	4	7
E	Two or more fluid collections and/or retroperitoneal air	4	> 50	6	10

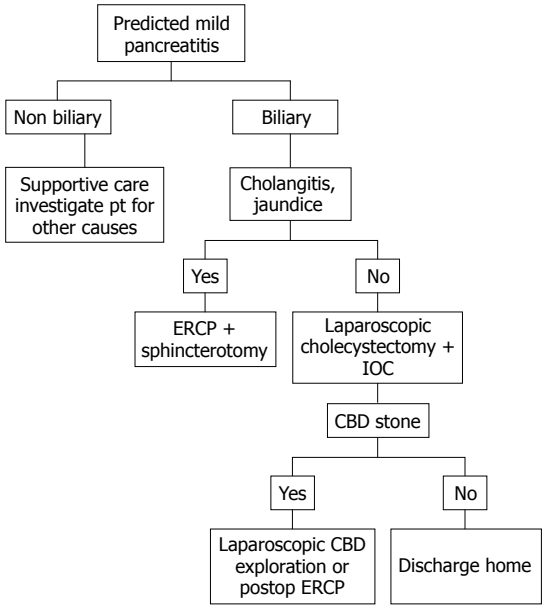

Figure 1 Algorithm for management of mild acute pancreatitis. IOC: Intraoperative cholangiography; CBD: Common bile duct.

be serious complications of fluid resuscitation especially in patients who have developed cardiovascular dysfunction or acute respiratory distress syndrome.

Adequate pain control is essential. Parenteral analgesia is usually needed with an advantage in using patient-controlled analgesia. Opiates are normally used including morphine and meperidine. Since there are no studies directly comparing the effects of meperidine or morphine on sphincter of Oddi manometry, morphine seems to provide more benefit by offering a longer duration of action and fewer side effects[73].

Supplemental oxygen is required in all patients. The oxygen saturation should be maintained at 95% or higher with supplemental oxygen administered by nasal cannula or by a face mask in order to prevent pancreatic necrosis.

The role of activated protease in producing organ failure is not clear. Although antiprotease treatment has been successful in experimental pancreatitis, it has not been shown to offer a survival benefit, but only a reduction of the incidence of complications in human disease[74,75].

Since cytokines could play an important role in AP, many agents have been used in experimental animals to ameliorate or prevent the inappropriate activation of the immune systems.

Anti-TNF-α antibody has been shown to reduce the induction of further cytokines by inflammatory cells but the peak TNF-α production appears to be within 1 or 2 h of the onset of disease. This action can compromise the use of TNF-α blockers in the clinical setting since the presentation and diagnosis of disease occurs after the peak of TNF-α production[76-78].

The role of calcium channel signaling in cytokine release and systemic organ injury in AP has been reported in one animal study. In this study, Hughes demonstrated that calcium channel blockers were associated with a dramatic reduction of TNF-α release with an improvement in overall survival from 40% to 80% between untreated rats and animals pre-treated with diltiazem, respectively[79].

An interleukin-1 receptor antagonist has been shown to be associated with decreased severity of pancreatitis in animal models, however these results have not been translated into clinical practice so far[80,81].

Since acute lung injury in AP results in an up-regulation of vascular adhesion molecule-1 (VCAM-1) cell surface receptor expression on pulmonary vascular endothelium, and neutrophil sequestration, experimental studies showed that blocking VCAM-1 decreased lung injury in AP[82,83]. It is believed that a VCAM-1 antagonist can offer a therapeutic option to improve the systemic manifestation and therefore the prognosis of AP.

Administration of monoclonal antibodies directed against the adhesion molecule, junctional adhesion molecule C expressed by endothelial cells showed a significant reduction in secretion of inflammatory cytokines, and in acinar cell necrosis[84].

Recently, there have been reports considering 5-fluorouracil treatment more effective compared to single inflammatory cytokine blockers in modulation of inflammatory mediators in experimental AP[85]. However, although the last decade has seen an increase in experimental studies on cytokines, the clinical treatment of the disease remains unchanged.

MANAGEMENT OF SAP: "EARLY PHASE"

SAP is defined by the Atlanta classification as an AP with local and/or systemic complications[86]. Some patients develop pancreatitis-associated organ failure during the early phase of the SAP[87].

The initial management of SAP is supportive based on fluid resuscitation, analgesia and enteral nutrition.

All patients should have thrombo-prophylaxis with low molecular weight heparin; however the decision to begin stress ulcer prophylaxis is still debatable.

SAP induces a catabolic state. Hence, early nutritional support is essential in order to avoid malnutrition.

An important issue in the early treatment of SAP is the optimal delivery of nutrition. After initial enthusiasm towards parenteral nutrition (PN), recent guidelines advise early enteral nutrition (EN) through a nasojejunal tube[88]. Patients with AP are characterized by loss of the gut barrier function which is involved in both local and systemic infectious complications[89]. In a meta-analysis analyzing 5 randomized control trials since 1997, Petrov *et al*[90] showed that EN, compared to PN, had a statistically significant lower risk of infection and mortality. Although there are studies supporting a lower infection rate when the tube is positioned in the jejunum, Eatock *et al*[91] demonstrated that nasogastric feeding is as good as nasojejunal feeding.

Although the role of probiotics in AP has been investigated in different clinical trials and meta-analyses, in a recent randomized controlled trial Besselink *et al*[92] showed that, in patients with predicted SAP, probiotic prophylaxis did not reduce the risk of infectious complications and was even associated with an increased risk of death.

Although the main management in the early phase of SAP is advocated to be mainly conservative and supportive in order to avoid organ dysfunction, there are conditions such as gallstone pancreatitis for which early endoscopic or surgical intervention has to be sought.

The benefits of ERCP with sphincterotomy (ES) has been studied in 3 randomized trials[93-95] and 2 meta-analyses[96,97]. Patients with predicted mild acute biliary pancreatitis (ABP) in the absence of cholangitis have not shown benefits from an early ERCP.

The decision on management of patients with predicted severe ABP is still debatable. The most recent United Kingdom guidelines recommend that urgent therapeutic ERCP should be performed within 72 h of admission in all patients with predicted severe ABP, whether or not cholangitis is present[98].

However, a recent meta-analysis by Petrov *et al*[97] demonstrated that early ERCP with or without ES had no beneficial effect in patients with predicted mild or severe ABP without cholangitis. The conclusion of this study was partially supported by the 2007 guidelines of the American Gastroenterology Association which stated that early ERCP in patient with severe ABP without signs of acute cholangitis is still not uniformly accepted in the literature[99].

However, optimal timing of laparoscopic cholecystectomy (LC) in patients with ABP is still contentious. In mild ABP, LC with operative cholangiography has been considered the definitive treatment[100].

Early laparoscopic cholecystectomy (ELC) can be performed as soon as the serum amylase decreases and symptoms improve[101].

Heinrich *et al* recently analyzed 4 prospective trials evaluating the optimal timing for surgery and concluded that ELC should be preferred in patients with mild to moderate ABP while in patients with severe ABP who did not have surgery for necrotizing pancreatitis, cholecystectomy appears to be favorable after full recovery[101-105].

MANGEMENT OF SAP: "LATE PHASE"

The main life-threatening event which characterizes the late phase of SAP is infection of the necrotic pancreatic parenchyma. Infection tends to occur in 10% to 50% of patients with necrotizing pancreatitis and develops 2-3 wk after the onset of symptoms[26,106-108]. The mortality increases from 5%-25% in patients with sterile necrosis to 15%-28% when infection occurs[24,107,109-112].

The infection is thought to be caused by translocation of enteric flora from the small and large bowel, since gram-negative bacteria such as *Escherichia coli* has been the most common species isolated. The use of antibiotic prophylaxis has changed the microbiology in favor of gram-positive and fungal organisms such as *Staphylococcus* species and *Candida*[113-116].

The debate on prophylactic antibiotics in sterile necrotic pancreatitis is still open. Since 1991, when Bradley introduced the concept of conservative treatment of sterile necrotic pancreatitis with the use of antibiotics, different randomized, controlled trials were conducted in order to investigate the beneficial effects of antibiotic therapy[117]. Initial studies were conducted with imipenem, ciprofloxacin and metronidazole[118-121].

These studies showed that antibiotic prophylaxis in

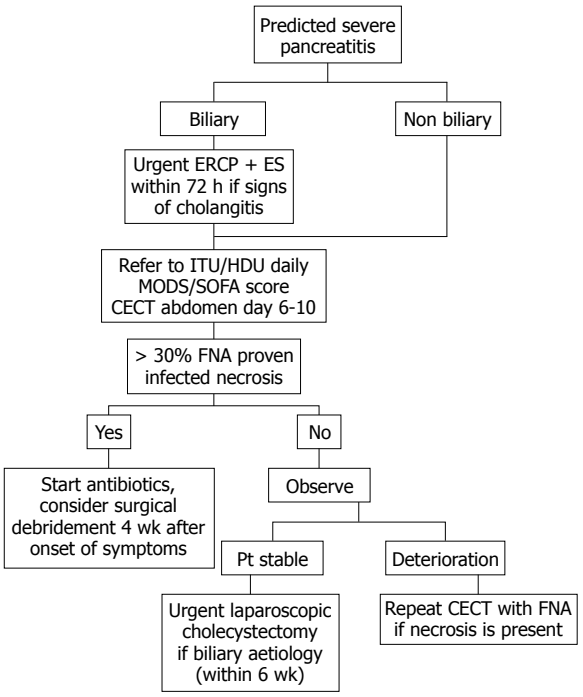

Figure 2 Algorithm for management of severe acute pancreatitis. ERCP: Endoscopic retrograde cholangiopancreatography; ES: ERCP with sphincterotomy; Pt: Patient; MODS: Multiple organ dysfunction score; SOFA: Sequential organ failure assessment; CECT: Contrast-enhanced computed tomography; FNA: Fine needle aspiration.

SAP is capable of reducing the incidence of bacterial infection of pancreatic necrosis but it was unable to improve hospital mortality.

Two placebo-controlled, double-blind trials by Isenmann and Dellinger demonstrated a lack of significant benefit of prophylactic antibiotics on infection and surgical intervention rates[122,123]. However, a recent Cochrane meta-analysis, which included the Isenmann study, showed a reduction in mortality with the use of β-lactams, although there was no evidence of a reduction in the pancreatic necrosis infection rate[124].

Despite the requirement for further multicenter double-blind studies, the use of prophylactic antibiotics in patients with proven necrotic pancreatitis on CT has been advocated. β-lactam agents are preferred to quinolones and the length of the therapy has to be at least 2 wk (Figure 2).

SURGICAL INTERVENTION

Surgery is considered the gold standard treatment for proven infected pancreatic necrosis[125].

Since Bradley has introduced the concept of conservative treatment in non-infected pancreatic necrosis, the timing of surgical intervention has changed substantially[117]. In the past, early surgical intervention was advocated and had a mortality rate up to 65%[126,127]. Recent studies demonstrated the benefit of postponing surgical intervention in reducing the mortality rate to 27%, allowing the immune system to demarcate the pancreatic necrosis[128,129].

The IAP (International Association of Pancreatology) Guidelines recommend that a patient with infected necrotic pancreatitis has to undergo surgery in the 3rd or 4th wk after the onset of symptoms[88]. However, postponing surgical intervention in necrotic pancreatitis can lead to prolonged use of antibiotics and an increased antibiotic resistance and higher incidence of *Candida* infection[130,131].

In a recent retrospective study, Besselink strongly advised avoidance of surgical intervention in the first 14 d even in the presence of multiple organ failure, and withholding of necrosectomy until day 30[132].

The aim of surgical management is to remove all the pancreatic tissue with necrosis in order to reduce the release of inflammatory mediators. Necrosectomy and drainage of infected acute necrotizing pancreatitis can be performed with different approaches such as radiological, endoscopical and surgical intervention.

Radiologic necrosectomy

Since Freeny presented the first series of patients with infected necrotic pancreatitis treated with only CT-guided percutaneous catheter drainage in 1998, this approach has not been considered very successful in debriding thick necrotic pancreatic tissue[133-135]. However, a combination of percutaneous catheter drainage and intensive care support can offer an alternative to surgery[136]. In unstable patients, percutaneous catheter drainage can be successful in draining pus and reducing the systemic manifestations of sepsis and in doing so, prepare the patient for surgery[133].

The placement of the catheter can be obtained through an anterior or retroperitoneal approach. To achieve an adequate drainage of the thick pancreatic necrotic collection, catheters with multiple side holes with a minimum diameter of 12-14 Fr are required[137]. Percutaneous drainage can lead to suprainfection of the pancreatic collection due to colonization of the indwelling catheter. However, clinical infection has been shown to be unlikely if all material is drained within 2-3 d.

Endoscopic necrosectomy

The endoscopic approach with the help of endoscopic US (EUS) has been reported to be effective in recent retrospective studies[138-141]. This technique can be considered as a natural orifice transluminal endoscopic surgery (NOTES) procedure since it employs a transenteric access route.

The most common access is through the posterior wall of the stomach under EUS and under Doppler imaging in order to avoid vascularized areas. The procedure is normally performed under sedation and requires good endoscopic skills.

Initially, a trans-gastric puncture under EUS guidance is performed using a 19-gauge needle through which a guidewire is passed into the cavity and coiled multiple times under fluoroscopic vision. The tract is dilated by a dilator which is passed over the guide and further dilatations can be done using 10-15 mm balloons. This dilation will allow an optimal insertion of the upper endoscope into the pancreatic cavity. The necrosectomy

is performed using different devices such as polypectomy snare, transparent cap baskets and different graspers.

The debridement of necrotic tissue is followed by copious irrigation with normal saline solution. A pig-tail drain inserted into the cavity and a nasocystic drain are used for continuous lavage. The lavage has to be repeated until all the necrotic material is removed[139,140]. The use of antibiotics is advised until the nasocystic drain has been removed.

Although optimal necrosectomy is normally achieved with multiple sessions, in a recent retrospective study which included 6 consecutive patients, Mathew *et al*[139] demonstrated that complete removal of necrotic tissue can be achieved with a single procedure. In this study none of the patients had procedure-related complications; however injury to visceral and vascular structures must be borne in mind as potential life-threatening complications.

Recent data from the literature indicated that the endoscopic approach could be a safe and effective modality for management of infected pancreatic necrosis with potentially lower morbidity than the traditional surgical approach. However, the data was from small prospective studies and multicenter prospective randomized trials are needed to clarify the role of the endoscopic approach in necrotic pancreatitis.

Surgical necrosectomy

The aim of surgery is to control the focus of infection through removal of necrotic tissue. This is based on preserving the endocrine and exocrine functions of the pancreas and allowing postoperative removal of retroperitoneal debris and exudates.

Until recently, necrosectomy was generally performed only by an open route. In order to achieve postoperative continuous drainage of debris, 4 different techniques have been advocated[142]: (1) open necrosectomy with open packing and planned re-laparotomy[143-149]; (2) open necrosectomy with planned re-laparotomy, staged and repeated lavage[150-153]; (3) open necrosectomy with continuous lavage of the lesser sac and retroperitoneum[153-161]; (4) open necrosectomy with closed packing[162].

Open necrosectomy with open packing and planned re-laparotomy: The abdominal cavity is filled with non-adherent packing. Successive laparotomies are performed every 48 h for further debridement which can be performed in intensive care under sedation. The abdomen is closed with drains when granulation tissue appears.

Open necrosectomy with planned re-laparotomy, staged and repeated lavage: After a primary necrosectomy repeated debridement is performed on alternate days until all necrotic tissue has been replaced by granulation tissue. To improve the surgical access some surgeons use an abdominal wall zip.

Open necrosectomy with continuous lavage of the lesser sac and retroperitoneum: Primary necrosectomy is followed by continuous closed lavage of the retroperitoneum. The procedure is based on insertion of 2 or more double 20-24 French drains and a single lumen 28-38 French silicone rubber on each side of the abdomen and placed with the tip at the tail of the pancreas. The smaller lumen of the drains is used for the inflow of the lavage and the larger lumen for the outflow. At least 35-50 L lavage are requested in the first days. Drains can be removed after 2-3 wk.

Open necrosectomy with closed packing: After the removal of the necrotic tissue, the residual cavity is filled with gauze-packed Penrose drains and suction drains. Drains are removed after 7 d.

Of these techniques open necrosectomy with closed continuous lavage of the lesser sac and the retroperitoneum seems to have the lowest mortality and it is the most common approach used by surgeons.

In specialized centers, open surgical management of infected necrosis can reduced the mortality from 80% to 10%-20%. This high mortality has induced surgeons to find a new, less invasive approach in order to reduce the activation of an inflammatory response in patients who are already seriously ill.

MINIMAL INVASIVE SURGICAL APPROACH

Over the last 2 decades, the role of minimal invasive surgical approaches to necrotic pancreatitis has increased. Minimal invasive techniques can be classified under 2 groups: (1) video-assisted retroperitoneal debridement (VARD) and (2) laparoscopic transperitoneal debridement (LTPD).

VARD

This approach was first described by Carter *et al*[163] in 2000 using an operative nephroscope inserted over an 8 Fr pigtail catheter, positioned under CT guidance. Since this initial experience, different studies have performed necrosectomy through a retroperitoneal approach using a rigid nephroscope or zero degree laparoscope.

This technique is performed by placing the patient in a supine position with his left flank elevated. A subcostal or intercostal incision is made to follow the retroperitoneal drains up to the necrotic area which is opened bluntly through digital examination. Necrotic tissue is debrided by a ring forceps and a suction device. A 10 mm laparoscope and a long 10 mm trocar are inserted through the retroperitoneal wound. Under laparoscopic vision, the remaining necrotic tissue is removed by laparoscopic graspers. Two surgical drains are inserted into the necrotic cavity via the incision[164-170].

Recently, Bucher *et al*[170] described a new technique based on a single laparoscopic port in order to avoid repeated necrosectomies. Using a 5 mm laparoscope with a 30 degree optic, a 12 mm trocar was placed in the drain tract. Through this big trocar, 5 mm instruments were used simultaneously with the 5 mm laparoscope. The necrotic cavity was dilated by insufflation with CO_2 at a

pressure of 8 mmHg in order to avoid potential bacterial translocation. At the end the procedure, the cavity was inspected with a 30 degree 10 mm laparoscope. Among 8 patients who underwent this new procedure only one did not have a successful debridement and had repeated necrosectomy.

The VARD approach has the great advantage of avoiding peritoneal contamination but is limited in necrosis extraction, and the need for repeated sessions is quite common. Other limitations of VARD are its low ability in detecting colonic ischemia, and in performing cholecystectomy or insertion of a feeding jejunal tube at the time of debridement[141].

LTPD

Cuschieri described for the first time the technique of laparoscopic infracolic necrosectomy with irrigation of the lesser sac as a valid alternative to open necrosectomy[171].

Although this laparoscopic technique has been described in the literature through case reports since 2002[172-175], only recently Parehk reported a retrospective study on hand-assisted laparoscopic surgery for pancreatic necrosectomy[174]. This study described 18 patients with necrotizing pancreatitis who underwent laparoscopic necrosectomy using an infracolic approach to access the lesser sac with a hand access port in order to enlarge the window in the transverse mesocolon and to bluntly remove the necrotic tissue. The outcomes were encouraging with mean length of stay of 16.3 d after the procedure and a reduction in the incidence of major wound complications.

This technique gives a better exposure of the lesser sac, left paracolic gutter and head of the pancreas, apparently overcoming the main limitation of the retroperitoneal approach in not debriding necrotic tissue completely. On the other hand the transperitoneal approach carries the risk of peritoneal contamination with infected necrosis.

Despite the use of less invasive techniques, complications following debridement of necrotic pancreatic tissue are still common. Pancreatic or enterocutaneous fistulae occur in 30% of patients and it seems related to the severity and extent of the underline necrosis[176]. Fistulae should be managed conservatively initially, deferring surgical closure of fistulae until pancreatitis is completely resolved. Other early complications are wound infection and wound dehiscence which seems less common with the minimal invasive approach. Postoperative bleeding is normally treated through an endovascular procedure with the help of an interventional radiologist.

Late complications such as pancreatic insufficiency and development of organized sterile necrosis are also common[177].

CONCLUSION

Although most patients with AP will have a benign outcome, it is crucial to assess the patient using multi-factorial scoring systems and inflammatory markers in order to evaluate the severity of the condition.

Early management should be conservative, based on fluid resuscitation with a focus on maintaining optimal organ perfusion. Managing the patient in the ICU should be contemplated when patients show signs of clinical deterioration.

A CT scan should be performed at least 48 h after the onset of symptoms and is considered the gold standard for diagnosis of necrotic pancreatitis. As part of the conservative intensive treatment, nasojejunal feeding is recommended in order to reduce bacterial translocation.

In ABP, early ERCP in patients without signs of acute cholangitis is not recommended. Infected necrotizing pancreatitis is the main indication for surgical debridement. The best time of surgery is 3-4 wk after the onset of the condition.

The last 2 decades have seen the emergence of new minimal invasive approaches in performing surgical debridement. However, no randomized controlled studies have been published in comparing different techniques.

Nevertheless, the results of the PANTER trial, a prospective multi-institutional randomized study comparing VARD versus open laparotomy, are still awaited[178].

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EDITORIAL

Systemic abnormalities in liver disease

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Abstract

Systemic abnormalities often occur in patients with liver disease. In particular, cardiopulmonary or renal diseases accompanied by advanced liver disease can be serious and may determine the quality of life and prognosis of patients. Therefore, both hepatologists and non-hepatologists should pay attention to such abnormalities in the management of patients with liver diseases.

Key words: Systemic abnormality; Risk of surgery; Drug dosage; Liver disease

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INTRODUCTION

Systemic abnormalities are often seen in liver disease, possibly because of the following characteristics of the liver as an organ[1]. (1) The liver is the key organ for the metabolism of protein, carbohydrate and fat. The liver produces many proteins, including coagulation factors. (2) The liver is a major organ for drug metabolism and the removal of hormones and other substances. (3) The liver is frequently invaded by various pathogens, and hepatitis virus infection causes not only serious liver diseases, but also exhibits extrahepatic manifestations through either immunological or non-immunological mechanisms. (4) The liver has a high blood flow supplied from the portal vein and the hepatic artery, leading to changes in portal, systemic or cardiopulmonary circulation in severe or advanced liver disease. (5) The liver is a major hematopoietic organ in the fetus and produces hematopoietic growth factors such as thrombopoietin[2].

In this review, in addition to systemic abnormalities often found in liver disease, dosage adjustment of drugs and risks of surgery in patients with liver disease are summarized. These factors should always be considered in the diagnosis or management of patients with liver disease.

CARDIOPULMONARY SYSTEM IN LIVER DISEASES

Patients with advanced liver disease have mild hypoxemia attributable to an alteration in ventilation-perfusion matching[3]. When a patient with liver disease exhibits dyspnea in the absence of detectable primary cardiopulmonary disease, hepatopulmonary syndrome (HPS) or portopulmonary hypertension (PPHTN) should be considered[4]. Clinical features and diagnostic criteria for HPS and PPHTN are shown in Table 1.

Hepatopulmonary syndrome

HPS is defined as the triad of liver disease, pulmonary gas exchange abnormalities leading to arterial deoxygenation and evidence of intrapulmonary vascular dilatation (IPVD)[4-7]. Although the mechanism for the development of HPS is complex and unclear, ventilation-perfusion mismatch and enhanced production of nitric oxide in the lung have been proposed as the predominant mechanisms[7].

A diagnosis of HPS requires demonstration of IPVD and an increased alveolar-arterial oxygen difference (AaDO2) (> 15 mmHg[8] or > 20 mmHg[6,9])

Table 1 Difference between HPS and PPHTN modified from Rodríguez-Roisin *et al*[4]

	HPS	PPHTN
Prevalence	11%-32% of patients with liver cirrhosis	2% of patients with portal hypertension
Pathogenesis	Increased intrapulmonary shunting	Unknown
Intrapulmonary vascular dilatations	(+)	(-)
Pulmonary arterial hypertension	(-)	(+)
Symptom	Dyspnea, platypnea	Dyspnea on exertion, syncope, chest pain
Clinical manifestations	Cyanosis	No cyanosis
	Orthodeoxia	Accentuated pulmonary component of Ⅱs
	Spider nevi	Systolic murmur, edema
ECG findings	None	RVH, RBBB, right axis deviation
Arterial blood gas levels	Moderate-to-severe hypoxemia (< 60-80 mmHg)	No/mild hypoxemia
Chest radiography	Normal	Cardiomegaly, hilar enlargement
CEE	Positive finding; left atrial opacification for > 3-6 heart beats after right atrial opacification	Usually negative finding
^{99m}TcMAA shunting index	⩾ 6%	< 6%
Pulmonary hemodynamics	Normal/low PVR	Elevated PVR mPAP > 25 mmHg at rest or > 30 mmHg with exercise
OLT	Indicated in severe stages	Only indicated in mild-to-moderate stages

HPS: Hepatopulmonary syndrome; PPHTN: Portopulmonary hypertension; RVH: Right ventricle hypertrophy; Ⅱs: Second heart sound; ECG: Electrocardiography; RBBB: Right bundle-branch block; $AaPO_2$: Alveolar arterial oxygen gradient; CEE: Contrast-enhanced echocardiography; ^{99m}TcMAA: Technetium-99m-labelled macroaggregated albumin; PVR: Pulmonary vascular resistance; mPAP: Mean pulmonary artery pressure; OLT: Orthotopic liver transplantation.

on breathing of air at room temperature and pressure. In order to diagnose IPVD, trans-thoracic contrast-enhanced echocardiography[4], technetium-99m-labeled macroaggregated albumin scanning[10] and pulmonary arteriography are useful. HPS is reported to occur in 11%-32% of patients with chronic liver disease, mainly among those with liver cirrhosis[11-15]. HPS patients who experience severe hypoxemia at rest should receive continuous long-term low-flow oxygen therapy, although no data on its long-term effectiveness is available. Complete resolution of HPS following orthotopic liver transplantation (OLT) has been observed in > 80% of reported cases[16]. At present, OLT appears to be the most effective therapy for patients with HPS.

Portopulmonary hypertension

PPHTN refers to pulmonary arterial hypertension that is associated with portal hypertension. PPHTN should be suspected in hypoxemic patients without pulmonary vascular dilatation[4,7,17]. The most common symptoms are dyspnea on exertion, syncope and chest pain. Although the cause of PPHTN is unknown, it has been reported that vasoactive substances could reach the pulmonary circulation at abnormally high concentrations owing to portosystemic shunts or decreased hepatic metabolism, leading to the pathological pulmonary vascular lesions exhibited in PPHTN[4,18]. Severe PPHTN is a contraindication of OLT, which can result in perioperative death from acute right ventricular failure[19,20]. Therefore, it is very important to distinguish between HPS and PPHTN in patients who are candidates for liver transplantation[4,7,21].

Idiopathic pulmonary fibrosis

Chronic hepatitis C virus (HCV) infection has been associated with a variety of extrahepatic complications, and an association between idiopathic pulmonary fibrosis (IPF) and HCV infection has been reported[22]. Studies undertaken in Japan and Italy suggest that the incidence of anti-HCV antibody positivity in patients with IPF is significantly higher than in those without IPF[22,23]. Arase *et al*[24] reported that the cumulative rate of IPF development is 0.9% at 20 years after HCV infection, which is significantly higher than that after hepatitis B virus (HBV) infection.

Coronary artery disease

It has been reported that atherosclerosis-related coronary artery disease (CAD) is less frequent in patients with liver cirrhosis than in controls matched with cirrhotic patients according to age, sex or cigarette smoking[25-28]. The prevalence of myocardial infarction was found to be significantly lower in cirrhotic patients than that in non-cirrhotic patients (1.7% *vs* 6.4%)[28]. The mechanisms of this protective effect on coronary atherosclerosis are unclear, but it may be associated with liver-related cholesterol metabolism and hematologic changes in cirrhotic patients. In contrast to the reported effect of cirrhosis on CAD, Alyan *et al*[29] demonstrated that HCV infection is an independent predictor of increased coronary atherosclerosis when patients with severe liver disease and cirrhosis are excluded from the analysis. It is thus suggested that the incidence of coronary atherosclerosis in patients with liver disease could be influenced by the severity of the liver disease and by hepatitis virus infection.

Recently, nonalcoholic steatohepatitis (NASH) has become well known as one of the leading causes of cirrhosis, and NASH is known to be strongly related to insulin resistance[30]. Kadayifci *et al*[31] reported that the prevalence of all CAD risk factors and metabolic syndrome was significantly higher in NASH-related cirrhotic patients than in cirrhotic patients with other etiologies.

High cardiac output and increased circulatory volume

In patients with advanced cirrhosis, cardiac output and circulatory volume are increased because systemic vascular peripheral resistance is reduced and oxygen consumption is decreased by arteriovenous shunting[32,33]. However, it is also known that these circulatory changes rarely result in heart failure.

Cardiomyopathy

Dilated and hypertrophic cardiomyopathies associated with HCV infection have been reported[34-36]. Although HCV has been isolated from the myocardium of patients with myocarditis and cardiomyopathy[37], there are conflicting reports on the incidence of HCV infection in these patients[38-41]. International epidemiological studies examining the incidence of HCV infection in patients with non-ischemic cardiomyopathy should be conducted. Some HLA haplotypes have been identified in a particular population of patients with HCV-associated cardiomyopathy, suggesting that genetic predisposition may be involved in the development of the disease[42]. Although the mechanisms of myocardial damage by HCV have not been characterized, HCV core protein may damage the myocardium either directly or indirectly through immunological mechanisms[43-45]. The treatment of chronic hepatitis C with interferon is sometimes contraindicated in patients with myocardial dysfunction[46]. Therefore, understanding and assessing cardiomyopathy as an extrahepatic manifestation of HCV infection is important.

ENDOCRINE SYSTEM IN LIVER DISEASES

Because the liver is involved in the synthesis and metabolism of many kinds of hormones, various abnormalities of circulating hormone levels are found in patients with advanced liver disease. HCV infection is known to be linked to some endocrine disorders.

Diabetes mellitus

Type-2 diabetes mellitus is present in more patients with chronic HCV infection than in those with HBV infection (21% and 12% in the United States, 23.6% and 9.4% in the United Kingdom, respectively)[47-50]. The association between HCV infection and diabetes mellitus is thought to be responsible for insulin resistance, which is shown to be directly induced by HCV core proteins[51]. Insulin resistance is also reported to be closely associated with the progression of fibrosis[52]. Diabetes mellitus is considered to increase the risk of the development of hepatocellular carcinoma and to increase mortality[53,54]. The monitoring of blood glucose should be performed on patients with chronic HCV infection, and in particular on those with advanced fibrosis, and appropriate treatment may be required when diabetes mellitus is diagnosed. The treatment of diabetes should be performed carefully owing to existing liver damage and hepatotoxicity of oral hypoglycemic drugs, particularly in cirrhotic patients. Oral hypoglycemic drugs, like biguanides which improve insulin resistance, for advanced liver diseases may be useful because insulin resistance is considered to be the main cause of glucose intolerance in patients with advanced liver disease[55]. α-glucosidase inhibitors may also be effective, because they reduce the absorption of glucose from the intestine and improve postprandial hyperglycemia[56]. Insulin treatment should be carefully performed in cirrhotic patients, because insulin resistance is increased and the metabolism of insulin decreases as liver diseases advance[55].

Thyroid disease

Thyroid disease often accompanies chronic HCV infection, particularly in elderly women (13% in patients with HCV infection)[56]. Anti-thyroid autoantibodies are also found in patients with chronic HCV infection (0% in men and 14.7% in women)[57]. Interferon-α therapy has been independently associated with the development of thyroid disorders[58,59]. Therefore, thyroid function or anti-thyroid autoantibodies should be evaluated before and during anti-viral treatment with interferon. Furthermore, thyroid cancer is also reported in patients with chronic HCV infection, particularly in those with thyroid autoimmunity[60,61].

Nonalcoholic fatty liver disease (NAFLD)

NAFLD is the most common cause of chronic liver disease in the western world, and NAFLD is known to be accompanied by type-2 diabetes and hyperlipidemia in about 30% of patients[62]. In addition, endocrine disorders such as hypopituitarism or hypothyroidism have been associated with NAFLD (the prevalence is reported to be 2.3% and 15% in NAFLD cases, respectively), although the precise mechanism for this association is unknown[62-64]. Patients with NAFLD accompanied by hypopituitarism may be susceptible to central obesity, dyslipidemia and insulin resistance, leading to disease progression.

HEMATOLOGICAL ABNORMALITIES IN LIVER DISEASES

Erythrocyte abnormalities

About 50%-75% of patients with chronic liver disease develop anemia by various mechanisms[65] including hemodilution, hypersplenism, myelosuppression caused by viral infection or hemolysis (by either immunological or non-immunological mechanisms). Alcoholism has a close association with low dietary intake of folate and vitamin B12, and is known to inhibit the absorption of both nutrients from the gut leading to macrocytic anemia[66,67]. Alcoholic liver cirrhosis is associated with spur cell hemolytic anemia[68], although a recent report revealed that this can also be induced by NASH[69]. Other anemia-causing factors found in liver diseases include hemolysis in Wilson disease, alcoholic liver disease (Zieve syndrome), pregnancy (HELLP syndrome) and myelosuppression followed by viral hepatitis[65].

Leukocyte abnormalities

Leukocytopenia, especially neutropenia, is often found in advanced chronic liver disease. Although splenic sequestration of leukocytes due to hypersplenism or serum hematopoietic progenitor inhibitory factors has been thought to be the main mechanism causing leukocytopenia[70,71], a shortened neutrophil lifespan caused by increased apoptosis may also be responsible[72]. Lymphocytopenia is also often found in patients with liver cirrhosis, possibly due to malnutrition[73]. In contrast, HCV infection can cause monoclonal proliferation of B lymphocytes leading to the induction of various autoimmune disorders[74], and an increased association with the development of non-Hodgkin's lymphoma has been reported[75]. Interestingly, the functional maturation of B lymphocytes has been proven to occur in the livers of patients with hepatitis C[76].

Platelet abnormalities

Patients with chronic liver disease show a reduction in both number and function of platelets[65], and platelet count is shown to decrease with disease progression, especially in the case of HCV infection. The aspartate aminotransferase to platelet ratio index has recently been reported to be a useful predictor of the progression of liver fibrosis in HBV infection[77] and recurrent HCV infection after liver transplantation[78]. The mechanisms responsible for the decrease in platelet count in chronic liver disease include splenic platelet sequestration due to hypersplenism[79] and a reduced level or activity of thrombopoietin[80], which is a hematopoietic factor for thrombocytes produced by mature hepatocytes[2]. In chronic HCV infection, bone marrow suppression by HCV itself[81], or immune-mediated destruction of platelets through production of anti-platelet antibody or formation of immune complexes[81], has been reported to be the cause of thrombocytopenia. Because platelet-associated immunoglobulin (Ig) G is found in as many as 88% of hepatitis C patients[82], it is not unusual for hepatitis C patients with thrombocytopenia to be diagnosed with idiopathic thrombocytopenic purpura.

With regard to platelet function, deficiency in platelet aggregation[83] or platelet-vessel wall interaction[84] has been reported in patients with cirrhosis, resulting in a tendency to bleed profusely.

Recently, the platelet count to spleen diameter ratio has proven to be effective for ruling out the presence of esophageal varices[85]. Therefore, platelet count is an important parameter for assessing disease progression and the presence of complications in advanced liver disease.

Abnormal coagulation

Because most coagulation factors are only synthesized in the liver[86], liver damage can easily lead to abnormal coagulation or a tendency to bleed profusely. Moreover, disseminated intravascular coagulation (DIC) is often seen in seriously ill patients with conditions including sepsis, malignancies and liver failure. Since both liver failure and DIC present with prolonged prothrombin time (PT), it is sometimes difficult to distinguish the two. It has been reported that a decrease in factor Ⅷ (not synthesized in the liver) and decreasing fibrinogen levels and platelet counts over time could indicate DIC accompanying liver failure rather than liver failure alone[87]. Levels of factor V are also reported to be useful for distinguishing the two conditions, since they are < 10% of normal levels in DIC and 10%-40% of normal levels in cirrhosis[87]. Vitamin K deficiency can also cause prolonged PT, but in contrast to liver failure and DIC, it leads to near normal levels of factor V[87].

In addition to prolonged PT, liver diseases are associated with hyperfibrinolysis[88], dysfibrinogenemia[89], endothelial dysfunction[90] and low count and/or decreased function of platelets[81,83], which all increase the risk of profuse bleeding in patients with advanced liver diseases.

GASTROENTEROLOGICAL ABNORMALITIES IN LIVER DISEASES

The liver has a unique anatomical characteristic in that a large amount of blood is supplied directly to the organ from the intestines *via* the portal vein. Consequently, various complications of the gastrointestinal tract are seen in advanced liver diseases.

Portal hypertensive gastropathy and esophageal varices

In a recent large-scale study, 37% of patients with HCV infection and advanced fibrosis in the liver were found to have portal hypertensive gastropathy (PHG)[91]. Biochemical markers of liver disease severity such as low serum albumin, high bilirubin or low platelet count may be correlated with the prevalence of PHG[91]. The presence of PHG may be predictive of esophageal varices[91], and low platelet count could be an indicator of the development of varices[92]. Some previous reports suggested that patients with primary biliary cirrhosis (PBC) may develop esophageal varices at a relatively early stage of the disease when other symptoms related to cirrhosis are not exhibited[93,94]. In recent studies, it has been recommended that PBC patients with a decreased platelet count (140 000-200 000/mm^3) should be screened for esophageal varices[95,96]. Nonselective beta-blockers may be effective for the treatment of PHG[92].

Pancreatic and biliary cancer

In a recent report, it was found that past exposure to HBV may be associated with the development of pancreatic or biliary tract cancer[97,98]. Anti-HBc antibody tests were positive in 7.6% of pancreatic cancer cases and 3.2% of controls[97], and a 2.4-fold increased risk of extrahepatic bile duct cancer in chronic HBV infection was reported[98]. HBV DNA integration in these tissues may have a pathogenetic influence. However, it is debatable whether the risk of biliary or pancreatic cancers is increased by HCV infection[98,99].

SKIN LESIONS IN LIVER DISEASES

Vascular spiders and palmar erythema are well known as skin lesions in patients with liver cirrhosis[100]. These skin lesions are thought to be related to excess estrogen, although the level of serum estrogen has been reported to be normal in patients with liver cirrhosis[100,101].

Chronic hepatitis infection is thought to be associated with various extrahepatic manifestations such as cutaneous lesions[102,103], including mixed cryoglobulinemia, porphyria cutanea tarda (PCT), lichen planus (LP), pruritus and urticaria. When these skin lesions are found, hepatitis virus infection should be considered.

Mixed cryoglobulinemia

Mixed cryoglobulin contains both a polyclonal IgG and a monoclonal IgM rheumatoid factor, and about 80% of mixed cryoglobulinemia is associated with HCV infection. Diagnosis of mixed cryoglobulinemia is made by skin palpable purpura, low serum complement levels and detection of circulating cryoglobulin[104-107]. Palpable purpura is a major finding suggestive of vasculitis. It is reported that a reduction in HCV load by treatment with interferon[108-110] could decrease serum cryoglobulin levels and improve skin lesions.

Porphyria cutanea tarda

PCT is caused by reduced activity of the enzyme uroporphyrinogen decarboxylase, and exposure to the sun can induce the development of skin erythema, vesicles and bullae[111]. A strong association between sporadic PCT and HCV infection has been reported, and a systematic review showed HCV infection in about 50% of patients with sporadic PCT[112]. Chronic HCV infection may impair porphyrin metabolism and cause sporadic PCT, but the mechanism for this is unclear.

Lichen planus

Although LP is a relatively rare skin disorder, it can be seen in patients with chronic liver diseases, particularly those with HCV infection[113]. It has been reported that anti-HCV antibodies are present in 10%-40% of these patients, but the relationship between HCV infection and LP is still uncertain[113,114]. During interferon treatment for chronic HCV infection, the development or exacerbation of LP has been reported[115]. In contrast, the improvement of oral LP in HCV-infected patients treated with interferon has also been reported[116].

Gianotti-crosti syndrome (GCS)

GCS is characterized by a symmetric papular eruption, which is mainly observed on the cheeks, buttocks and extensor surfaces of the forearms and legs[117]. GCS usually occurs in association with several viral infections, and acute HBV infection has been reported to be one of the most common causes of GCS in infants and young children[118,119]. It is, however, reported that GCS rarely occurs in adults[120].

RENAL DISEASES ASSOCIATED WITH LIVER DISEASES

Renal diseases in patients with liver disease can be classified into two major categories by etiology. One is hepatitis virus-associated nephropathy including membranous nephropathy, membranoproliferative glomerulonephritis (MPGN) and mesangioproliferative glomerulonephritis[121-123]. The other is hepato-renal syndrome (HRS), which is a serious complication of advanced liver cirrhosis[124]. When a patient with chronic liver disease has proteinuria and/or hematuria, hepatitis virus-associated nephropathy should be considered (Figure 1)[122].

Hepatitis virus-associated nephropathy

HBV infection may be directly associated with a variety of renal diseases, including membranous nephropathy and MPGN[121,125]. The diagnosis is based on an assessment of the status of HBV replication (HBeAg/Ab and HBV DNA levels)[126], laboratory findings (urinalysis and liver function test) and a kidney biopsy, although it is sometimes difficult to detect the deposition of viral antigens in the kidney by routine immunohistochemical analysis. HBV-associated nephrotic syndrome due to membranous nephropathy is not uncommon in children, and spontaneous recovery has been reported, which is often associated with seroconversion of HBeAg to anti-HBe[127]. In adults, on the other hand, spontaneous resolution is relatively uncommon and antiviral therapy may be effective[128].

HBV-related MPGN is characterized by the deposition of antigen-antibody complexes in the mesangium and subendothelial space. Antiviral therapy with interferon[129,130] or lamivudine[131] has been reported to induce remission in HBV-associated MPGN.

HCV infection is more often associated with renal diseases such as mixed cryoglobulinemia, MPGN and membranous nephropathy[122] than is HBV infection. The prevalence of MPGN in patients with HCV infection is higher than that of patients with HBV infection[123]. A high incidence of mixed cryoglobulinemia (35%-90%) has been reported in patients with HCV infection[132-134]. Mixed cryoglobulinemia is a systemic vasculitis and can frequently cause renal disease. MPGN associated with mixed cryoglobulinemia is the predominant type of glomerulonephritis, and the incidence of MPGN in patients with mixed cryoglobulinemia is approximately 30%[123,135,136]. The pathogenesis of HCV-related cryoglobulinemic MPGN is unknown, but the glomerular damage may be caused by the deposition of immune complexes of HCV, and IgG and IgM rheumatoid factors[135]. The clinical manifestations of renal diseases may include hematuria, nephritic range proteinuria and renal insufficiency. HCV-infected patients should be screened for proteinuria, hematuria, hypertension and renal function, as well as for cryoglobulinemia, complement and rheumatoid factors[124]. A kidney biopsy should be performed when

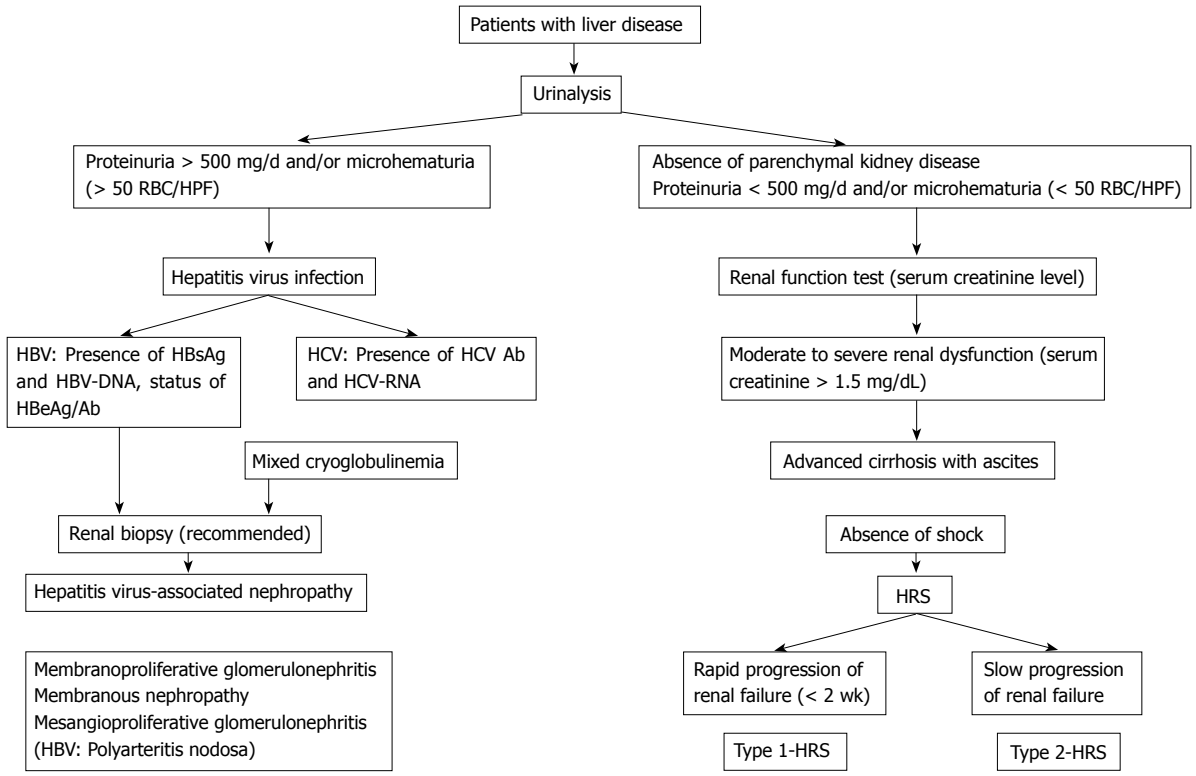

Figure 1 Diagnostic strategy for renal disorders found in patients with liver disease. RBC: Red blood cell; HPF: High power field; HBV: Hepatitis B virus; HCV: Hepatitis C virus.

Table 2 New diagnostic criteria for hepatorenal syndrome in cirrhosis[146]

Cirrhosis with ascites
Serum creatinine > 133 μmol/L (1.5 mg/dL)
No improvement of serum creatinine level (decrease to ≤ 133 μmol/L) after at least 2 d with diuretic withdrawal and volume expansion with albumin. The recommended dose of albumin is 1 g/kg of body weight per day up to a maximum of 100 g/d
Absence of shock
No current or recent treatment with nephrotoxic drugs
Absence of parenchymal kidney disease as indicated by proteinuria > 500 mg/d, microhematuria (> 50 red blood cells per high-power field) and/or abnormal renal ultrasonography

significant proteinuria and/or impaired renal function are observed (Figure 1).

There have been many reports on beneficial responses of patients with HCV-induced renal disease to antiviral therapy with interferon[137-139]. Improvements in both serum cryoglobulin levels and plasma creatinine concentration have been reported in patients who exhibited undetectable levels of serum HCV RNA after interferon therapy. A recently developed combination therapy involving pegylated interferon and ribavirin improved the rate of sustained virologic clearance. It has also been reported that this combination regimen improved HCV-associated mixed cryoglobulinemia[140,141], although the efficacy of ribavirin is limited when renal insufficiency is complicated. Recently, the effectiveness of anti-CD20 chimeric monoclonal antibody (rituximab) in the treatment of cryoglobulinemic glomerulonephritis has been reported[142].

HRS

HRS involves renal failure in patients with severe liver disease in the absence of any identifiable renal pathology[124,143,144]. The incidence of HRS in patients with cirrhosis and ascites is 18% and 39% after 1 and 5 years of follow-up, respectively[145]. New criteria for a diagnosis of HRS were reported by the International Ascites Club in 2007 (Table 2)[146]. HRS may be classified into two types: type-1 HRS is characterized by a rapid progression of renal failure (within 2 wk) with the mortality rate at 2 wk being about 80%. In contrast, the degree of renal failure is less severe in patients with type-2 HRS, and median survival is around 4-6 mo[144]. Type-1 HRS is often induced by a precipitating event, in particular spontaneous bacterial peritonitis[147]. HRS is related to renal vasoconstriction following a reduction of effective circulating volume due to peripheral vasodilation[148]. OLT is the ideal treatment for cirrhotic patients with HRS, but the survival rate after OLT is lower in those patients than in patients without HRS (60% *vs* 70%-80% at three years)[149]. The combined use of vasoconstrictors and albumin is one of the most useful options in treatment of patients

with HRS[150].

The diagnostic strategy for hepatitis virus-associated nephropathy and HRS is shown in Figure 1.

NUTRITIONAL ABNORMALITIES IN LIVER DISEASES

Protein-energy malnutrition is often found in patients with liver cirrhosis, and is reported to have an incidence as high as 30%-90%[151]. Malnutrition has been reported to be associated with survival and surgical outcome in cirrhotic patients, and nutritional intervention such as supplementation of branched-chain amino acids (BCAA) could improve patient outcome[152,153]. Hypermetabolism, which is indicated by increased resting energy expenditure, has been reported to be associated with malnutrition, and the measurement of energy metabolism could thus be used to predict the prognosis of liver cirrhosis[154,155]. Hypermetabolism may be explained by increased α-adrenergic activity[156], and further investigation is required to determine whether the administration of beta-blockers is effective for the treatment of malnutrition in patients with liver cirrhosis.

The administration of BCAA has been reported to not only reduce malnutrition and improve energy metabolism, but also to improve the liver function and quality of life of patients with cirrhosis[157-159]. However, BCCA supplementation may be harmful because glucose intolerance can be exhibited by cirrhotic livers[55]. The administration of BCAA and α-glucosidase inhibitors in combination may improve the therapeutic effects[160].

In addition, hepatic glycogen stores decrease in patients with liver cirrhosis because of liver atrophy, leading to the development of a severe catabolic state after fasting. Late evening snacks including BCAA are recommended in order to avoid such problems during the night-time[158,159].

NERVOUS SYSTEM IN LIVER DISEASES

Liver diseases frequently affect the nervous system.

Hepatic encephalopathy

Hepatic encephalopathy (HE) is a major complication of chronic liver diseases with neuropsychiatric manifestations ranging from sleep disturbance to deep coma. The swelling of astrocytes and oxidative stress induced by ammonia, inflammatory cytokines, benzodiazepines and hyponatremia have been regarded as essential causes of HE[161]. Psychometric tests such as the number connection test may be effective for assessing HE[162], and neuropsychiatric function should be carefully evaluated in patients with chronic liver diseases including alcoholic liver disease and Wilson disease (WD). Minimal hepatic encephalopathy (MHE) occurs in 30%-80% of cirrhotic patients, which could be a serious problem because of the associated impaired quality of life[161,163]. For the management of patients with HE, removal of precipitating causes, such as gastrointestinal bleeding, excessive protein intake, hypokalemic alkalosis, infection, constipation (which can all increase blood ammonia levels), hypovolemia, hypoglycemia, hypoxia and the administration of sedatives, is important. Lactulose is the most effective therapy for HE so far, and has been reported to even be effective for patients with MHE[164].

Polyneuropathy and neurocognitive dysfunction

Nervous system disorders such as neuropathy, fatigue and depression are often associated with chronic HCV infection, even without advanced cirrhosis[165-167]. Mixed cryoglobulinemia in HCV causes peripheral neuropathy as a moderate axonal sensory polyneuropathy[168], and chronic sensory polyneuropathies were found in 13% of HCV infected patients with cryoglobulinemia in southern Italy[169]. In addition, paresthesia has been found in about 20% of patients with chronic HCV infection, particularly among those with cryoglobulinemia[170,171]. The therapeutic response to antiviral treatment for neuropathies is generally unsatisfactory[172]. Furthermore, fatigue or depression is found in many patients with chronic HCV infection, with incidences of about 50% and 35%, respectively[173-175]. These neurocognitive dysfunctions have been characterized epidemiologically or pathophysiologically in chronic HCV infection[166,167,176], and may be explained by the neuroinvasion of HCV, because HCV has been reported to be found in monocytes/microglia of the central nervous system[176,177]. However, antiviral treatment with interferon, particularly interferon-α, is known to exacerbate depression[178].

Guillain-Barre syndrome

Guillain-Barre syndrome accompanying hepatitis virus infection, which is primarily associated with HBV and rarely with HCV or hepatitis A virus, has also been reported[179-181]. Immune complexes have been found in the serum of these patients, which may be the cause of the development of the disease.

Autonomic and sensory nerve dysfunction

Autonomic and sensory nerve dysfunction presenting as reduced 24 h heart rate variability and lower current perception threshold has also been reported to be common in patients with PBC, particularly those suffering a severe form of the disease for a long period[182]. Peripheral sensory nerve dysfunction may contribute to hyperesthesia, leading to the itching that is a characteristic symptom of PBC[182].

Wilson disease

WD is an autosomal recessive inherited disorder of copper metabolism, resulting in excessive accumulation of copper in virtually all organs, especially in the liver. The clinical spectrum of liver diseases in WD is broad from asymptomatic with only mild biochemical

abnormalities, chronic active hepatitis, liver cirrhosis to fulminant hepatitis[183]. Copper accumulation is also remarkable in the cornea (Kayser-Fleischer rings) and brain in patients with WD. The patients show various neuropsychiatric symptoms such as dysarthria, dyspraxia, ataxia, a tremor-rigidity syndrome and psychoses, and progressive extrapyramidal neurological disorder is the typical sign of neurologic WD[183,184]. The initial neurological symptoms usually develop in mid-teenage years or in the twenties and are frequently misdiagnosed as behavioral problems associated with puberty[184].

BONE DISEASE ASSOCIATED WITH LIVER DISEASES

Metabolic bone disease is a common complication of chronic liver disease, particularly in patients with end-stage liver disease due to cholestatic disorders such as PBC. It has been reported that osteoporosis occurs in approximately 20%-30% of patients with PBC[185,186], and older age and severity of the disease were the main risk factors for osteoporosis[187]. Moreover, there were 2-fold relative increases in the risk of bone fractures in patients with PBC compared with the general population[188]. Although the mechanisms responsible for osteoporosis in liver diseases are not well understood, cirrhotic patients show reduced osteoid thickness, osteoblast surfaces and bone formation rate, suggesting the presence of an osteoblast defect[189].

In post-liver transplantation populations, osteoporosis is known to be a major complication[190]. Several studies suggest that bone loss is remarkable within the first year after liver transplantation. The etiology is multifactorial, consisting of both preexisting low bone mineral density associated with underlying liver disease and post-transplantation factors[190]. High-dose corticosteroids and immunosuppressive agents such as cyclosporine A are thought to contribute to bone loss[191]. Although few prospective studies are available, vitamin D and calcium supplementation are generally recommended for those patients[192].

ARTHROPATHY IN LIVER DISEASES

Arthralgia or arthritis is often seen in patients with liver diseases, and it is not rare that arthropathy may be the first presentation of the disease[193]. In acute viral hepatitis, especially in HBV infection, patients may show polyarthralgia or polyarthritis during the prodromal period and immune complex is thought to be responsible for the pathogenesis[194]. Similar arthropathies are seen in patients with chronic hepatitis C, autoimmune hepatitis, PBC, hemochromatosis or WD[193]. Arthralgia or arthritis is reported to be the most common extrahepatic manifestation in patients with hemochromatosis, autoimmune hepatitis and PBC. Polyarthralgia is also the most common symptom of mixed cryoglobulinemia[195], which often occurs secondary to HCV infection.

SEXUAL DYSFUNCTION IN LIVER DISEASES

Erectile dysfunction (ED) is frequently problematic in patients with chronic liver diseases such as hemochromatosis[196], alcoholic liver disease[197] or liver transplant patients[198], leading to worsening of quality of life in those patients. Hypogonadism or protein malnutrition found in patients with advanced liver disease may induce ED[199,200]. Removal of causative factors such as iron or ethanol, or administration of testosterone may improve ED in such patients[197,198]. ED is also more frequent in patients with chronic HCV infection than in control subjects (39% *vs* 14%, respectively) probably due to a direct effect of HCV on neurovascular and hormonal systems (i.e. low testosterone levels)[201]. Successful antiviral treatment such as interferon plus ribavirin may improve such sexual dysfunction in patients with chronic HCV infection[202].

DOSAGE ADJUSTMENT OF DRUGS IN LIVER DISEASES

The liver is the main organ of biotransformation and elimination of drugs. Thus, liver diseases could affect drug metabolism, resulting in abnormally high concentrations of drugs in the body.

Drug elimination by the liver may be determined mainly by first-pass effect, hepatic metabolism and biliary extraction. In addition, since the liver produces most plasma proteins, decreased liver function could influence the binding of drugs to plasma proteins, leading to changes in the distribution and elimination of such drugs[203].

The first-pass effect of each drug is variable and drugs with high first-pass effects are listed in Table 3[204]. The serum concentration of these drugs could easily be elevated by a decrease in hepatic blood flow (especially portal blood flow) or total hepatocyte mass. For drugs with a high first-pass effect, both initial dose and maintenance dose should be reduced in cirrhotic patients if the drug is orally administered.

Drug metabolism in the liver largely depends on the activity of the cytochrome P (CYP) 450 enzymes, which is known to be affected in different ways in patients with cirrhosis. The activities of CYP3A, 1A and 2C19 are reported to be substantially reduced, whereas those of CYP2D6, 2C9, 2B and 2E1 are also reduced, but to a lesser extent[205]. The severity of liver cirrhosis is estimated using Child-Pugh (C-P) classification, and patients with C-P grade A show mild to moderate deterioration of CYP activities. On the other hand, patients with C-P grade B or C are shown to have prominent reduction in CYP activity. Therefore, the doses of drugs mainly metabolized by CYP 3A, 1A or 2C19 in the liver may need to be reduced in these patients (Table 3)[206]. Moreover, cirrhotic patients often have impaired renal function, despite a normal serum

Table 3 Drugs that may need to be administered at reduced doses in patients with liver cirrhosis[187]

Drugs with high first-pass effect	Drugs metabolized mainly by		
	CYP 1A2	CYP3A4	CYP2C9
Amitriptyline	Acetaminophen	Quinidine	Diclofenac
Bromocriptine	Caffeine	Amiodarone	Ibuprofen
Diltiazem	Mexiletine	Lidocaine	Mefenamic acid
Flumazenil	(R)-Warfarin	Midazolam	Tolbutamide
Fluorouracil	Imipramine	Diazepam	Phenytoin
Imipramine	Theophylline	Amitriptyline	Phenobarbital
Isosorbide dinitrate	Propranolol	Imipramine	(S)-Warfarin
Labetalol	Tamoxifen	Carbamazepine	Losartan
Lidocaine	Estradiol	(R)-Warfarin	Piroxicam
Morphine		Erythromycin	
Nifedipine		Clarithromycin	
Pentazocine			
Propranolol			
Verapamil			

creatinine level[207]. Therefore, creatinine clearance should be measured or estimated in cirrhotic patients to determine the appropriate dose of drugs with predominant renal excretion.

RISK OF SURGERY IN PATIENTS WITH LIVER DISEASES

Patients with liver diseases face relatively high risks during surgery, and these risks could be increased according to the progression of liver disease. A decrease in hepatic blood flow during anesthesia or surgery is thought to be mainly responsible for postoperative liver damage, and Cowan *et al*[208] reported that a major reduction in hepatic blood flow occurs after the induction of anesthesia, but not during or after surgery.

In acute viral hepatitis, as well as acute alcoholic hepatitis, the risk of surgery might be extremely high. Therefore, surgery should be avoided unless the situation is life-threatening[209]. In contrast, surgery on patients with chronic hepatitis can generally be considered safe[210], and there have been no deaths or complications reported in patients with chronic hepatitis C undergoing laparoscopic cholecystectomy[211]. In patients with liver cirrhosis, complications and mortality rates of surgery are high[212], especially if one or more of the following factors apply to the patient: elevated bilirubin, prolonged PT, ascites, decreased albumin, encephalopathy, portal hypertension, elevated creatinine concentration, intraoperative hypotension and emergent surgery[213]. The outcome of surgery also depends on the invasiveness or duration of the operation[214]. There are two scores for the assessment of the progression of liver cirrhosis: the model for end-stage liver disease (MELD) score and the C-P grade[215]. Both scores are useful for assessing the risk of surgery, and C-P classification was reported to be useful in stratifying the risk of death. Two studies showed that patients in class A had mortality rates of about 10%, those in grade B had mortality rates of around 30%, and those in grade C had mortality rates above 70%[216,217]. A recent study in Italy also reported similar mortality rates of surgery for patients with liver cirrhosis (C-P grade A; 7.1%, C-P grade B; 23%, C-P grade C; 84%)[218]. Teh *et al*[219] recently reported that MELD score, age and American Society of Anesthesiologists class are important predictors for the risk of postoperative mortality in patients with cirrhosis. Interestingly, they also reported that the risk was not associated with the type of surgery performed. For MELD, operation risks increase according to the score, and one report showed that a MELD score $\geqslant$ 14 or plasma hemoglobin levels < 10 g/dL were independent predictors of poor outcomes in patients undergoing abdominal surgery excluding hepatic surgery[220]. For patients undergoing laparoscopic cholecystectomy, a preoperative MELD score of 8 was linked to high morbidity, and was suggested as the cutoff value for avoiding the operation in patients with liver cirrhosis[221]. However, Schiff *et al*[222] recently reported that preoperative platelet levels and PT (international normalized ratio) are more important factors for the safety of cholecystectomy than C-P grade.

In patients undergoing cardiac surgery, the risk of mortality or complications may be high when a cardiopulmonary bypass is performed on patients with chronic liver disease[223]. In general, the mortality of cirrhotic patients with C-P grade A is low, but that of patients exhibiting C-P grade B for a long period and that of all patients with C-P grade C is high, especially when open heart surgery is performed. Therefore, open heart surgery should be avoided for patients with C-P grade C, and cardiac operation on the beating heart is recommended for patients with C-P grade B[224]. The results of another study led to the recommendation that patients with a C-P score > 7 avoid cardiac surgery involving cardiopulmonary bypass[225].

In summary, surgery should be avoided for patients with acute hepatitis. However, surgery is generally safe for patients with chronic hepatitis and cirrhotic patients with C-P grade A. The risks are elevated for cirrhotic patients with C-P grade B or C, or patients with a MELD score of $\geqslant$ 8, though this might vary according to the type of surgery performed. Other predictive factors for safe surgery are platelet count and PT, both of which are markers for the tendency to bleed profusely and for the progression of liver disease.

CONCLUSION

Liver diseases often cause systemic abnormalities, and it is not uncommon that these complications, rather than the liver disease itself, determine the quality of life and prognosis of patients. Therefore, both hepatologists and non-hepatologists should always pay attention to the abnormalities caused by liver diseases, and should be concerned with their management in addition to the actual treatment of the liver disease itself.

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REVIEW

Eosinophilic colitis

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Abstract

Eosinophilic colitis (EC) is a rare form of primary eosinophilic gastrointestinal disease with a bimodal peak of prevalence in neonates and young adults. EC remains a little understood condition in contrast to the increasingly recognized eosinophilic esophagitis. Clinical presentation of EC is highly variable according to mucosal, transmural, or serosal predominance of inflammation. EC has a broad differential diagnosis because colon tissue eosinophilia often occurs in parasitic infection, drug-induced allergic reactions, inflammatory bowel disease, and various connective tissue disorders, which require thorough searching for secondary causes that may be specifically treated with antibiotics or dietary and drug elimination. Like eosinophilic gastrointestinal disease involving other segments of the gastrointestinal tract, EC responds very well to steroids that may be spared by using antihistamines, leukotriene inhibitors and biologics.

Key words: Eosinophilia; Colitis; Gastrointestinal disease; Ascites

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INTRODUCTION

Primary eosinophilic gastrointestinal disease (EGID), originally described by Kaijser in 1937[1], is a rare spectrum of gastrointestinal disorders characterized by inflammation rich in eosinophils, without evidence of known causes for eosinophilia, such as parasitic infection, drug reaction, or malignancy[2]. The disease can affect any segment or combination of segments of the gastrointestinal tract from the esophagus to the rectum, giving rise to various clinical presentations including eosinophilic esophagitis (EE), eosinophilic gastritis, eosinophilic gastroenteritis, and eosinophilic colitis (EC). Since secondary eosinophilic inflammation may occur in numerous gastrointestinal disorders such as IgE-mediated food allergy, gastroesophageal reflux disease, and inflammatory bowel disease, the true incidence and prevalence of primary EGID remains largely unknown. A recently established world-wide-web registry found that EGID mainly affects the pediatric population, although it has been reported in patients up to 68 years of age[3]. In the past few years, EE has been increasingly recognized as a distinct condition that affects about 1% of the population, and accounts for dysphagia and food impaction that remain non-responsive to traditional anti-reflux management, both in pediatric and adult gastroenterology[4]. Accordingly, several excellent reviews on EE have recently been published[4-6]. In contrast, EC represents the least frequent manifestation of EGID whether or not it presents with disease in other segments of the gastrointestinal tract[3]. EC appears to have a bimodal distribution that affects neonates with a relatively high prevalence and a separate group of young adults with no gender preference[2].

CLINICAL PRESENTATION

EGID in general has three hallmarks including peripheral eosinophilia (typically in the range of 5% to 35%), segmental eosinophilic infiltration of the

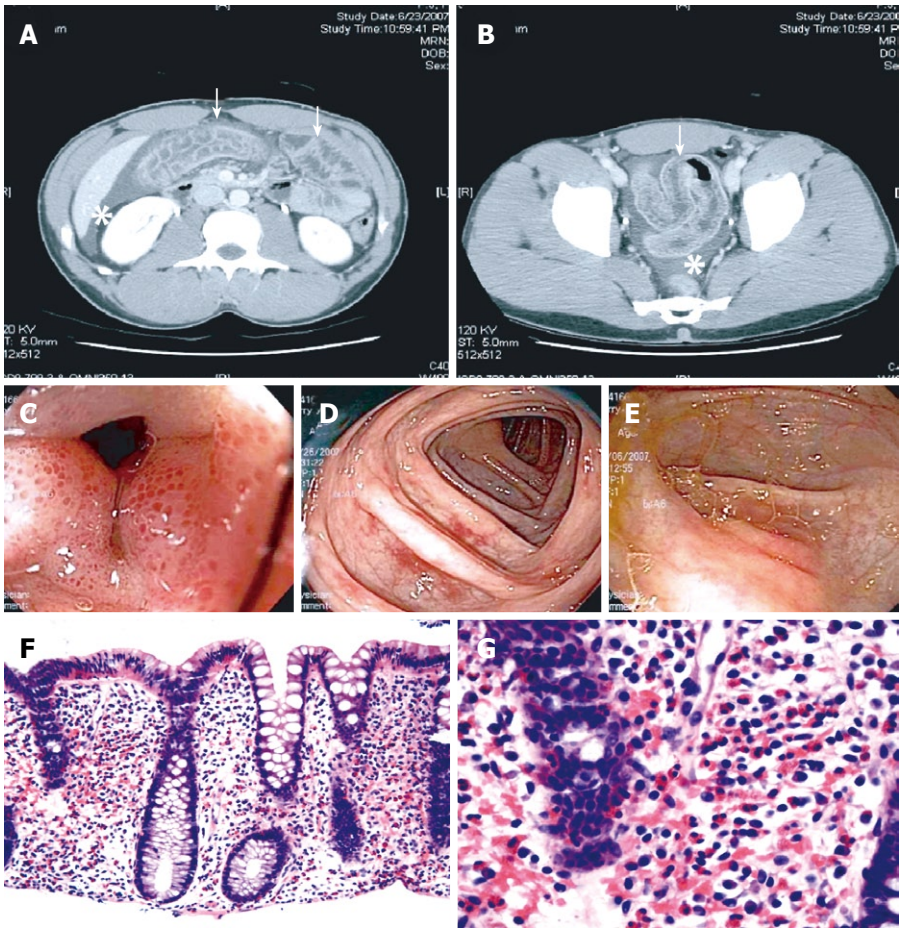

Figure 1 Diagnostic findings in EC. Representative images from a case of a previously healthy 30-year-old man with recurring episodes of abdominal pain, non-bloody diarrhea, and peripheral eosinophilia; extensive workup confirming EC by exclusion; and excellent response to short-term steroid therapy. A and B: Abdominal CT shows circumferential colon wall thickening (arrows) and moderate ascites (asterisks); C-E: Colonoscopy reveals patchy areas in the colon with mucosal edema and punctate erythema; F and G: Histology indicates markedly increased tissue eosinophilia in all examined segments of the colon. HE stains, magnification 100 × and 400 ×, respectively.

gastrointestinal tract, and functional abnormalities[7,8]. Importantly, up to 23% of patients with primary EGID have no peripheral eosinophilia[7]. Symptoms and signs of EGID are usually non-specific and, depending on the affected segment, include abdominal pain, nausea, vomiting, diarrhea, gastrointestinal bleeding, obstruction, malabsorption, weight loss, and ascites. In 1970, Klein *et al*[9] subdivided the disease based on the layer of intestinal wall most extensively infiltrated by eosinophils, to distinguish mucosa-predominant, muscularis-propria-predominant, and serosa-predominant forms of EGID.

The above classification provides good correlation of the physical symptoms and signs with the pathological findings, and it is also applicable to EC. Thus, mucosa-predominant disease shows evidence of mucosal dysfunction, such as protein-losing enteropathy, malabsorption, and diarrhea. Transmural disease is recognized by symptoms of intestinal obstruction and bowel wall thickening on imaging studies. Finally, serosal involvement is distinguished by the presence of eosinophilic ascites, with up to 88% eosinophils seen on fluid analysis[10]. Accordingly, while mucosal EC results in diarrhea[11], the transmural form has been associated with volvulus[12], intussusception[13,14], and even perforation[15,16], and involvement of the intestinal serosa may manifest with ascites[17], which was also illustrated by a case that we have encountered recently (Figure 1).

DIAGNOSTIC CRITERIA AND DIFFERENTIAL DIAGNOSIS

The diagnosis of EGID is made from the presence of gastrointestinal symptoms, peripheral eosinophilia, endoscopic and histological findings, and eosinophilic ascites, with no well-defined causes of eosinophilia on thorough evaluation[8]. A multidisciplinary task force has recently reached consensus on the diagnostic criteria of EE, including the presence of more than 15 eosinophils per high-power field in the esophageal squamous mucosa[6]. No such consensus exists for EC, although most authors have used a diagnostic threshold of 20 eosinophils per high-power field. Of note, normal values for tissue eosinophils vary widely between different segments of the colon, ranging from < 10 eosinophils per high-power field in the rectum to > 30 in the cecum[5], thus location of the biopsy is critically important for interpretation of findings.

More or less prominent tissue eosinophilia in the colon may result from a number of conditions (Table 1) and EC remains therefore a diagnosis of exclusion. Colonoscopic biopsies obtained from patients with inflammatory bowel disease, in particular with Crohn's colitis, often show severe tissue eosinophilia[18]. Parasitic infection of the colon with pinworms, roundworms, or whipworms may lead to marked eosinophilic infiltration, and repeated stool or serological testing may be needed to reveal this specific etiology[19-23]. Drug-induced EC has been de-

Table 1 Differential diagnosis of EC

Differential diagnosis of EC
Parasitic colitis
Enterobius vermicularis[19,20]
Strongyloides stercoralis[21,22]
Trichuris trichiura[23]
Drug-induced colitis
Clozapine[24]
Carbamazepine[25]
Rifampicin[26]
Non-steroidal anti-inflammatory drugs[27,28]
Tacrolimus[29]
Gold[30]
HES[35,51]
Inflammatory bowel disease[18]
Allogeneic bone marrow transplantation[33]
Tolosa-Hunt syndrome[34]

EC: Eosinophilic colitis; HES: Hypereosinophilic syndrome.

scribed in response to clozapine[24], carbamazepine[25], rifampicin[26], non-steroidal anti-inflammatory agents[27,28], tacrolimus[29], and gold[30]. EC has also been associated with autoimmune connective tissue disease including scleroderma, dermatomyositis and polymyositis[11,31,32], as well as with allogeneic bone marrow transplantation[33] and the rare Tolosa-Hunt syndrome that features inflammatory ophthalmoparesis[34]. The idiopathic hypereosinophilic syndrome (HES) may also affect the colon, but this rare condition presents with sustained and marked peripheral eosinophilia with end-organ damage that extends beyond the gastrointestinal tract (e.g. heart and skin)[35].

ETIOLOGY AND PATHOGENESIS

The etiology of primary EGID remains largely unknown. Several studies have suggested a relationship with specific food allergies; indeed, about 75% of affected patients have a history of allergy or atopy[2]. Cow's milk and soy proteins are the foods most frequently implicated in the infantile form of EC, although the condition has been described in infants exclusively breast-fed or given protein hydrolysate formulas[2]. Even less is known about the potential causes of the adult form of primary EC. A case report by Inamura *et al*[36] has demonstrated accumulation of mast cells in the colon interstitium after immunohistochemical staining for mast cell tryptase, which suggests the pathogenic role of IgE, while other observations suggest that EC may not be an IgE-mediated disease. Thus, colonic T cells in an animal model have been shown to transfer oral antigen-induced diarrhea to naive mice through a STAT6-dependent mechanism[37]. Specific eosinophil chemoattractants, such as interleukin-5 and eotaxins, may also have a pathogenic role in EC[38]. While EE may develop without other gastrointestinal involvement when experimental animals are sensitized and challenged in the lung, direct exposure of the gastrointestinal mucosa seems to result in multisegmental disease[39].

TREATMENT

No prospective randomized controlled trials exist to date on specific therapy for EC or any other forms of primary or idiopathic EGID. Therapeutic efforts have been based on case reports and small case series. Corticosteroid therapy has formed the backbone for initial management, and it has proven to be the most effective instrument for symptom control in EC[2,40,41]. Up to 90% of cases will respond within 2 wk of treatment, when a slow taper is initiated. However, relapse is frequent and requires recurrent courses or leads to steroid dependence. A role for budesonide has been demonstrated, particularly in disease of the right colon and ileum[42]. It must be emphasized that efforts to rule out parasitic or drug-induced EC are important since empiric treatment with corticosteroids may aggravate the patient's condition, or at least, it may be avoidable. The beneficial effect of elimination and elemental diets has been limited to cases with specific food allergies, especially in treating neonatal disease[43].

Approaches to avoid steroids by using alternative medications have been directed mostly to more prevalent forms of EGID, and expertise about their need and efficacy in EC has been limited. Antihistamine therapy in EGID appears to be gaining prominence. Ketotifen, an H1 antihistamine not yet available in US, has been shown to decrease symptoms as well as tissue eosinophilia[44,45]. The leukotriene inhibitor montelukast, an agent that blocks the action of potent eosinophil chemoattractant leukotriene D4, by competitively antagonizing its receptor expressed on eosinophils, has also been found to be helpful in EGID[46,47]. Mast cell stabilizers, such as cromolyn, are effective by inhibiting release of mast cell mediators such as histamine H1, platelet activating factor, and leukotoxin[48]. More recently, the role of biologics in EGID has also been studied, with favorable outcomes reported by using monoclonal antibodies targeting interleukin 5 (mepolizumab) and IgE (omalizumab)[49,50].

CONCLUSION

In summary, EC is a rare manifestation of the EGID spectrum, which does not appear to be increasing in prevalence, in contrast to recent trends seen in esophageal disease. This dichotomy suggests a disparate pathophysiology of EC, with the possibility that EC itself is a heterogeneous entity. While the pediatric form of EC often subsides without intervention, or upon withdrawal of atopic stimuli, the adult form may relapse and require short-term steroid therapy. Importantly, EC needs to be included in the differential diagnosis of many conditions with primary or secondary involvement of the gastrointestinal tract.

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REVIEW

Psychosocial stress and liver disease status

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Abstract

"Psychosocial stress" is an increasingly common concept in the challenging and highly-demanding modern society of today. Organic response to stress implicates two major components of the stress system, namely the hypothalamic-pituitary-adrenal axis and the sympathetic nervous system. Stress is anamnestically reported by patients during the course of disease, usually accompanied by a decline in their overall health status. As the mechanisms involving glucocorticoids and catecholamines have been deciphered, and their actions on immune cell function deeper understood, it has become clear that stress has an impact on hepatic inflammatory response. An increasing number of articles have approached the link between psychosocial stress and the negative evolution of hepatic diseases. This article reviews a number of studies on both human populations and animal models performed in recent years, all linking stress, mainly of psychosocial nature, and the evolution of three important liver-related pathological entities: viral hepatitis, cirrhosis and hepatocellular carcinoma.

Key words: Stress; Chronic viral hepatitis; Cirrhosis; Carcinoma; Hepatocellular; Liver pathology

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INTRODUCTION

Over time, stress has received a number of definitions in the scientific literature, more or less accurate or complete. One of the most commonly accepted psychological definitions has been that stress occurs when demands from the environment challenge an individual's adaptive capacity, or ability to cope[1]. Several life-changing or threatening events are considered to be "stressors"; factors that are either acute or chronic based on the duration of their interaction. Both have been associated with several immune system dysfunctions, whether or not the individual is affected by an acute or chronic disease[2].

Once an individual is subjected to such a stressor, specific pathways within the brain lead to the activation of the hypothalamic-pituitary-adrenal (HPA) axis as well as the central sympathetic outflow. This constitutes the stress response, releasing key peripheral mediators-glucocorticoids and catecholamines[3].

For a long time, it was suggested that stress influenced hepatic blood flow by inducing vasospasm and centrilobular hypoxia, leading to liver damage[4,5]. In more recent years, as the understanding of stress mediators has improved, the effect that stress has on the onset and development of liver damage during acute and chronic liver diseases has gained a new dimension[3,6].

This article reviews an important part of current literature on the effects of stress on the status of three major interrelated hepatic conditions: viral hepatitis, cirrhosis and hepatocellular carcinoma. We tried to cover the physiological aspects of the stress system and its relationship with several cellular pathways, the immune system effectors and the level of cellular alteration at the hepatic level.

THE HPA AXIS AND CENTRAL SYMPATHETIC NERVOUS SYSTEM

Physiology and interactions with immune-mediated inflammation

The HPA axis and the systemic sympathetic and adrenomedullary system are the key components of

the stress system. Their main function is to maintain basal and stress-related homeostasis[7,8]. They respond to several signaling molecules, such as cytokines produced by immune-mediated inflammatory reactions, tumor necrosis factor (TNF)-α, interleukin-1 (IL-1), and interleukin-6 (IL-6)[7-9].

Corticotrophin-releasing hormone (CRH) and noradrenergic neurons of the central stress system innervate and stimulate each other[7-10]. By using specific receptors, CRH stimulates norepinephrine secretion, while in turn norepinephrine stimulates CRH secretion primarily through α_1-noradrenergic receptors[7,8,10]. An ultrashort negative-feedback loop also exists for both CRH and norepinephrine, down-regulating their production. The serotonergic and cholinergic systems stimulate CRH, arginine vasopressin (AVP), and noradrenergic neurons whilst being inhibited by the opioid-peptide and γ-aminobutyric acid-benzodiazepine systems existing in the brain. Hypothalamic CRH neurons are inhibited by centrally secreted substance P while AVP neurons remain unaffected. Substance P also stimulates the central noradrenergic system[11-13].

CRH-induced secretion of proopiomelanocortin-derived and several other opioid peptides concurs with stress system activation[14,15] which enhances overall analgesia[7,8]. CRH also stimulates corticotrophin secretion through the corticotrophs of the anterior pituitary[16-18]. Adrenal medulla hormones, especially corticotrophin, are principal regulating factors of glucocorticoid secretion[19-23].

All key hormones secreted by HPA components play different roles in the modulation of the immune response and the development of the inflammatory reaction.

Some leukocyte functions are influenced by glucocorticoids, as they suppress cytokine immune activation by inhibiting the production of cytokines and other inflammation mediators, as well as causing resistance to cytokines[7,8,24,25]. The functionality of Type 1 helper T lymphocytes is suppressed whilst eosinophil apoptosis is stimulated. Adhesion molecule expression is inhibited along with their specific receptors[26], while the acute phase reaction is being potentiated[27]. The pituitary hormones, corticotrophin and β-endorphin[28,29], have immunopotentiating and proinflammatory properties. Additionally, β-endorphin produced at inflammatory sites is a potent local analgesic[30].

The hypothalamus exerts a regulatory effect over the HPA axis through CRH and AVP secretion. Their proinflammatory effects have been studied both *in vitro* and *in vivo*[31-38]. CRH concentrations remain high at inflammatory sites, while remaining undetectable in plasma samples obtained concurrently[31]. Rapid catabolism, uptake or binding are thought to be primary mechanisms which prevent CRH from remaining active in the systemic circulatory system[31,39,40]. In addition to the HPA axis, the central sympathetic nervous system is directly connected with the modulation of the stress response. Stimulation of the locus coeruleus-norepinephrine system activates the central nerve pathways, thus influencing peripheral sympathetic outflow[3,4]. This triggers catecholamine release from autonomic nerve endings and from the adrenal medulla[4]. Catecholamines can influence the hepatic inflammatory response by altering hemodynamics[3]. Recently, catecholamine receptors were discovered on immunocompetent cells, thus it is believed that catecholamines can directly influence the immune response[3,6].

STRESS AND CHRONIC VIRAL HEPATITIS

In recent years, a number of studies have established an increasingly clear link between psychosocial or psychophysical stress, personality types and the development of viral hepatitis. Both human clinical trials[41-46] and animal model studies[47-52] were devised in order to prove the interaction between the two.

A clinical trial, performed by Nagano *et al*[41] indicated a positive correlation between psychosocial stress and the severity of chronic hepatitis C. Type 1 personality subjects, due to the nature of their personality traits (low sense of control, object dependence of loss, unfulfilled need for acceptance and altruism) are highly likely to be affected by chronic psychosocial stress. Type 1 personality and psychosocial stress were positively linked to the severity of chronic hepatitis C. Stress was measured using a Stress Inventory questionnaire and a personality evaluation was devised using items from the Grossarth-Maticek theory (according to this theory, type-1 personality subjects are positively associated with malignant neoplasms and chronic diseases)[42]. Patients were divided into three groups depending on the severity of chronic hepatitis C, measured by liver function laboratory parameters (ALAT values, platelet count, albumin and total bilirubin levels). Ultimately, two distinct groups resulted by unifying the 2nd and 3rd initial groups, as their values were similarly elevated. Platelet count and serum albumin levels were positively correlated with hepatitis C severity, both of them being strongly correlated with elevated stress scores. ASAT values strongly correlated with both levels of stress and the presence of type 1 personality traits.

Kunkel *et al*[43] investigated the connections between depression scores, psychosocial stressors, social support, and biological markers of dysfunction of a group of 50 Korean immigrants with chronic viral hepatitis B. Several indicators of liver function, including hepatic transaminases, albumin levels, and prothrombin times (PT) were measured during routine clinic follow-up visits and were correlated with scores obtained from the short form Beck Depression Inventory (BDI-sf). Higher BDI-sf scores were significantly associated with elevated transaminases ($P < 0.001$). Both PT and decreased albumin levels were not significantly correlated with increased BDI-sf scores.

Both studies excluded patients with decompensated cirrhosis, malignant disease, coronary heart disease, stroke, co-infection or interferon treatment.

Clinical studies suggest that chronic psychosocial

stress affects antibody response after hepatitis B vaccination[43,44]. A higher score in stress inventory questionnaires given to the test subjects was positively correlated with a weaker immune response, when administering the same antigen dose[44,46].

Psychosocial stress has intricate relationships with inflammatory and fibrosing changes of the liver during the course of hepatitis[47]. Kaji et *al*[48] reported that serum levels of transaminases increased due to stress in the galactosamine-injured liver of rats. Several animal models have suggested that inflammation-related HPA-activated pathways are influenced by stress.

Psychophysical stress simulated by inescapable foot-shock induced elevations of glucocorticoids (GCs), exacerbating α-galactosylceramide-triggered apoptosis through proliferation of liver natural killer T (NKT) cells and up-regulating the expression of Fas antigen on hepatocytes. GC directly elevates Fas antigen expression, probably through intracellular signal cascades[49]. Several other studies[50,51] established that *in vivo* administration of dexamethasone, an exogenous GC, enhanced the number of liver α-galactosylceramide-activated Vα14 natural killer T cells in mice. As NKT cells play an important immunological role in liver homeostasis[52,53] as well as in hepatocyte apoptosis through their Fas-ligand coated surface[54-56], it is clear that GC elevations during stress negatively influence the pathological state of the liver.

Tamada *et al*[57] found in mice the increased production of IL-4-hepatic NK1.11 T cells after exogenous administration of dexamethasone, thus demonstrating that these cells are resistant to glucocorticoid-induced apoptosis. Their study suggested that the process may play a role in determining the hepatic Th1/Th2 balance in times of stress or during GC therapy.

GCs downregulate expression of endothelial cell adhesion molecules, thus producing inhibitory effects on neutrophil recruitment in liver[3,58]. Increased circulating endogenous GC levels hence decrease hepatic neutrophil chemotaxis, as well as lymphocyte recruitment[58].

GCs inhibit IL-6 and TNF-α at transcriptional and translational levels[59,60]. Endogenous corticosterone, at normal or stress levels, induces IL-6 and TNF-α in an *in situ* liver perfusion, which lead to the conclusion that GCs have additional non-suppressive effects[60].

STRESS AND LIVER CIRRHOSIS

Psychosocial stress *per se* may exaggerate inflammatory and fibrosing change in the cirrhotic liver[3,6,47].

Nagano *et al*[41] included in their clinical test a subset of patients affected by cirrhosis. They found the same positive correlation between psychosocial stress and liver injury, as ALAT values strongly correlated with high stress scores in the cirrhosis cohort.

Tanaka *et al*[61] conducted a long-term follow-up study determining risk factors for malignant transformation of cirrhotic lesions in Japanese patients. His study also demonstrated a possible link between the presence of stress and precipitating indicators for cirrhosis.

Several animal studies were conducted, outlining important cellular mechanisms that link stress response of the sympathetic nervous system, as well as alterations of the HPA axis responsiveness, with liver inflammation in cirrhosis.

Electric foot shock stress exacerbated liver injury in rats treated with carbon tetrachloride[62]. Alterations of the HPA axis responsiveness, as well as elevated plasma cytokine levels, accompany experimental chronic liver disease in mice. Elevated TNF-α and IL-6 levels, coupled with liver injury, decrease hypothalamic mRNA and protein expression of CRH. When mice are exposed to psychological stress, HPA axis functions abnormally, suffering defective activation and significant attenuation in the resultant release of GCs, compared to control groups[63].

NKT cell activity was linked with fibrosing and inflammatory damage in cirrhosis[64]. Epinephrine and norepinephrine, *via* several subtypes of adrenoceptor (AdR), cause expansion of liver NKT cells[49-52,65,66], production of IL-6 from hepatocytes and TNF-α[3,6,49] from Kupffer cells, as well as impairment of hepatic blood flow (HBF). GCs inhibit the production of IL-10, IL-6, TNF-α, PGE_2 leukotrienes and nitric oxide[3,6,61,66] from Kupffer cells. Two mechanisms are involved, a direct one (affecting the stability of mRNA and gene transcription) and an indirect one, by inhibiting the production of the nuclear factor (NF)-κB and the activator protein (AP)-1.

Tjandra *et al*[67] suggested that psychosocial stress itself can influence the course of hepatic inflammation, by directly altering IL-6 and TNF-α production.

Also, Kitamura *et al*[68] studied how immobilization stress can induce increased IL-6 mRNA expression in the liver, as well as an elevation of the plasma IL-6 level. He made a clear distinction between IL-6 produced within hepatocytes and that produced in non-parenchymal cells, using immunohistochemical techniques.

This distinction was further demonstrated by an *in vitro* experiment, using primary cultured rat hepatocytes[68]. This study also explored the effect sympathetic nervous system mediators such as epinephrine and norepinephrine have on TNF-α and IL-6 produced by Kupffer cells and hepatocytes. An increase in norepinephrine is mediated through α_1-, α_2- and β_1- adrenergic receptors, leading to an increased production of pro-inflammatory cytokines[68-72].

Nakajima *et al*[73] discovered that patients with liver cirrhosis and decreased NKT cell activity were at a higher risk of developing hepatocellular carcinoma than those with normal natural killer cell activity.

INFLUENCE OF STRESS ON HEPATOCELLULAR CARCINOMA (HCC) PROGNOSIS

The effects psychosocial stress has on immune suppression in patients with malignant metaplasia are well

established[74]. However, its effects on hepatocarcinoma are yet to be determined. It has been hypothesized that several psychosocial factors, including stress, may account in part for rapid hepatocarcinoma development. Biobehavioral models were suggested in order to demonstrate this interaction[74,75]. Cancers with a strong immune-mediation component, such as HBV-related HCC are believed to be the most appropriate[75].

Several studies proved the correlation between stress and progression of various types of cancer in humans[76-79]. In these studies, positive correlations were found between stress and the grade of dysplasia[76], overall survival[77,78] and cancer recurrence[79].

By using this knowledge, psychological intervention was used as a tool to improve immune functioning and to reduce progression[80-82]. Similar studies were conducted by Spiegel *et al* who observed extended survival in patients affected by cancer after group therapy and other forms of stress-relieving techniques[83].

Carcinoma is associated with high concentrations of TNF-α. Stress, as outlined in several studies, directly influences TNF-α, IL-1, and IL-6 expression[59-61,66-68], in turn influencing the activity of NKT cells[48,50,51]. As a result, stress and depression can influence tumor progression at a cellular level.

Several animal models demonstrated relationships between stress, immune reactivity and tumor growth, by influencing IL-2 production, plasma L3T4 antigen and elevating GCs[74,75].

Liu *et al*[83] demonstrated in a recent study that survival time of mice affected by HCC is greatly reduced when subjected to social isolation stress. He compared the titer of antibody to sheep red blood cell (SRBC), as well as IL-2 levels, and survival time between two groups. Individuals exposed to isolation stress positively correlated with a lower survival time as well as with negatively altered serum values of both SRBC antibodies and IL-2.

Psychosocial stress was also linked to increased DNA damage, alterations in DNA repair and inhibition of apoptosis[84-88].

Sivonova *et al*[84] studied how academic stress during student examinations positively affects oxidative stress and induces direct DNA damage. Single strand breaks of DNA as well as sensitivity to lipid oxidation and the antioxidant status were studied on examination day, as well as at a random time between examinations. They found that in stressful conditions oxidative damage to DNA as well as sensitivity to lipid oxidation were significantly increased ($P < 0.05$), while plasma antioxidant activity was severely decreased ($P < 0.05$), in comparison to the control group of non-stressed individuals.

Glaser *et al*[85,86] proved the association between rotational stress and low concentrations of O6-methyltransferase, an enzyme linked to DNA repair induced in response to carcinogen damage. Their animal study was conducted on forty-four rats to whom dimethylnitrosamine was administered. The group was divided into two, half being randomly assigned to a rotational stress condition[85].

In a study regarding psychological stress, Tomei *et al*[89] found that cellular death decreased during examination compared with a control group, after phorbol ester (tumor promoter through activation of protein kinase C[90]) inhibition of radiation-induced apoptosis. This goes on to demonstrate that stress, having a negative impact on apoptosis, serves as a tumoral proliferation promoter.

CONCLUSION

As seen above, stress has been identified in recent years as an important factor in the progression and outcome of several important liver pathologies. It influences the immune system and several intra- and inter-cellular mechanisms. Comprehensive models that try to integrate these complex mechanisms are being developed.

From a clinical perspective, a better understanding of how stress alters hepatic inflammation would provide additional tools for the management of important liver diseases. It would influence the quality of life of patients by shortening hospitalization times and ensuring a correct therapeutic approach.

For a better understanding of the relationship between stress and liver pathology, we suggest that further studies on both human and animal models be conducted. Comprehensive clinical trials could be devised, which would test positive correlations between elevated stress scores and a number of both serological and imaging parameters assessing disease in hepatic patients. Animal studies should investigate immunohistochemical and genetic alterations at cellular level in the liver of stress-challenged hepatic-impaired rodents.

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Pluronic L-81 ameliorates diabetic symptoms in *db/db* mice through transcriptional regulation of microsomal triglyceride transfer protein

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Abstract

AIM: To test whether oral L-81 treatment could improve the condition of mice with diabetes and to investigate how L-81 regulates microsomal triglyceride transfer protein (MTP) activity in the liver.

METHODS: Genetically diabetic (*db*/*db*) mice were fed on chow supplemented with or without L-81 for 4 wk. The body weight, plasma glucose level, plasma lipid profile, and adipocyte volume of the *db*/*db* mice were assessed after treatment. Toxicity of L-81 was also evaluated. To understand the molecular mechanism, HepG2 cells were treated with L-81 and the effects on apolipoprotein B (apoB) secretion and mRNA level of the MTP gene were assessed.

RESULTS: Treatment of *db*/*db* mice with L-81 significantly reduced and nearly normalized their body weight, hyperphagia and polydipsia. L-81 also markedly decreased the fasting plasma glucose level, improved glucose tolerance, and attenuated the elevated levels of plasma cholesterol and triglyceride. At the effective dosage, little toxicity was observed. Treatment of HepG2 cells with L-81 not only inhibited apoB secretion, but also significantly decreased the mRNA level of the MTP gene. Similar to the action of insulin, L-81 exerted its effect on the MTP promoter.

CONCLUSION: L-81 represents a promising candidate in the development of a selective insulin-mimetic molecule and an anti-diabetic agent.

Key words: Pluronic L-81; *db*/*db* mice; Animal models; diabetes; Microsomal triglyceride protein

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INTRODUCTION

Pluronic® surfactants or poloxamers are synthetic copolymers based on ethylene oxide and propylene oxide. They are synthesized by controlled addition of propylene oxide to the two hydroxyl groups of propylene glycol[1]. Pluronic surfactants are widely used in industries as defoaming and antifoaming agents in dishwashing, antifreeze, cutting and grinding fluids, water treatment, *etc.* They are also being investigated as drug delivery vectors[2] and cancer therapies[3]. Pluronic L-81

(L-81) contains 10% hydrophilic and 90% hydrophobic polyoxyethylene residues with a molecular weight of 2750.

L-81 has profound effects on the lipid metabolism of the intestine and the liver. Chronic feeding of rats with L-81 greatly reduced the lymphatic lipid transport from their intestine, without affecting the digestion and absorption of lipid into enterocytes. The absorbed lipid was however accumulated in the enterocytes, suggesting that L-81 interferes with lipoprotein assembly and/or exit of lipoproteins from the mucosal cells[4]. L-81 inhibits chylomicron formation but not very low density lipoprotein (VLDL) assembly in the intestine[5]. On the other hand, L-81 effectively inhibits VLDL and low-density lipoprotein (LDL) secretion in hepatocytes[6]. Assembly of VLDL in hepatocytes depends very much on the endoplasmic reticulum-residing protein microsomal triglyceride transfer protein (MTP). There is evidence that L-81 inhibits MTP activity in hepatocytes[7]. Consistent with these molecular effects, rodents fed with L-81 exhibited obvious weight loss, which is reversible upon withdrawal of the compound in the diet[4,8]. Because the inhibitory effect of L-81 on lipid transport is rapid and readily reversible, it is an attractive drug for controlling obesity.

Obesity, defined as a body mass index exceeding 30 kg/m^2, is epidemic in many developed countries. For instance, more than 50% of the adults in the USA are either overweight or obese[9]. Obesity is a strong risk factor for the development of insulin resistance and type 2 diabetes[10]. The risk of developing type 2 diabetes increases in parallel with increasing severity of overweight and obesity[11]. On the other hand, weight reduction is associated with a decreased incidence of type 2 diabetes[12]. Obesity is also strongly associated with cardiovascular diseases and cancers[13]. Clearly, it would be of great clinical benefit if effective prevention and treatments for obesity and associated type 2 diabetes were established.

Although L-81 is a potent anti-obesity drug, its potential in alleviating obesity-induced insulin resistance and type 2 diabetes has not been fully explored. Here we aimed to test whether L-81 could ameliorate diabetic symptoms using a mouse model of type 2 diabetes, *db/db* mice. *db/db* mice have a mutant leptin receptor which results in high plasma triglyceride and cholesterol levels. *db/db* mice develop significant obesity, fasting hyperglycemia and hyperinsulinemia within 6 wk of age[14]. We also investigated the possibility that L-81 affects MTP activity through transcription regulation.

MATERIALS AND METHODS

Cell culture and measurement of apoB secretion rates in HepG2 cells

HepG2 cells were obtained from the American Type Culture Collection (ATCC) and maintained in basal medium (MEM supplemented with 1.5 g/L sodium bicarbonate, 2 mmol/L glutamate, 2 mmol/L sodium pyruvate) with 10% FBS. In a typical experiment, cells were seeded into 6-well (35 mm) culture plates, allowed to grow to 70% confluence, and then incubated with 3 mL of either the control medium (basal medium supplemented with 3% BSA) or experimental media (control medium plus test substances) at 37℃ for the indicated time. At the end of the experiments, media were collected and analyzed for apolipoprotein B (apoB) and apoA-I by ELISA as described previously[15].

Measurement of human MTP and actin mRNA levels

Total RNA was isolated from HepG2 cells by the guanidinium thiocyanate method and the relative levels of the MTP large subunit and β-actin mRNA were determined by the DNA excess solution hybridization assays as described previously[15].

MTP promoter construct, transfection and reporter assay

The promoter-luciferase construct (MTP-250) which contains a 336-bp fragment encompassing position -250 to +86 of the human MTP promoter was generated by PCR as described in our earlier study[16] and cloned into promoterless pGL3-Basic vector (Promega, Madison, WI). For transfection, HepG2 cells were grown overnight (70% confluent) in 6-well plates and washed twice with serum-free medium. DNA-lipofectAMINE 2000 complexes, containing 1 μg MTP promoter-firefly luciferase construct, 0.1 μg pRL-SV40 renilla luciferase control vector, and 2 μg lipofectAMINE 2000 (Invitrogen) in 200 μL serum-free medium in each well, were allowed to form at room temperature for 30 min. The cells were then overlaid with the complex for 6 h at 37℃. After 16 h of recovery in complete culture medium, the cells were washed twice with serum-free medium, and experimental media with or without the indicated concentration of Pluronic L-81 were subsequently added. After 24 h, the cells were washed twice with ice-cool PBS and treated with passive lysis buffer (Pormega, Madison, WI). The lysates were assayed for both luciferase activities using the Dual-luciferase assay kit (Pormega, Madison, WI) according to the manufacturer's instructions. Luciferase activities were determined by Lumat LB 9507 luminometer (Berthold).

Animals and L-81 treatment

Male and genetically diabetic BKS·Cg-m +/+ Lepr^{db} (*db/db*) mice and their non-diabetic littermates C57BLKS/J (BKS) (5-6 wk of age; n = 3 mice per group) were obtained from Jackson Laboratories (Bar Harbor, ME). They were housed in environmentally controlled conditions with a 12-h light/dark cycle. Mice were fed a standard rodent chow diet (powdered) and water *ad libitum* in sterile cages. Animals were gathered together for 2 wk before the commencement of the experiment. Pluronic L-81 was kindly provided by BASF Corporation (Parsippany, NJ). To prepare food with the indicated amount of L-81 for treatment, L-81 was first dissolved in ethanol and sprayed on the powdered chow. The food was then air-dried to remove the carrier ethanol before it was used to feed the mice. Various parameters of the

mice including body weight, food and water intake were monitored on a regular basis as indicated.

Animal blood sampling, metabolic measurements, intraperitoneal glucose tolerance test (IPGTT), and histological analysis

Animals (n = 3 mice per group) were fasted for 5 h before blood was sampled from the retro-orbital sinus. Plasma glucose, insulin, adiponectin, triglycerides, cholesterol, alkaline phosphatase (ALP), alanine aminotransferase (ALT) levels were measured using standard enzyme assay kits. For IPGTT, mice were first fasted for 5 h and then received an intraperitoneal administration of glucose (2 g/kg). Blood glucose levels were determined using the One-touch Ultra Blood Glucose Monitoring System (LifeScan Inc) from the tail blood samples at 0 (before glucose administration), 30, 60, 90, and 120 min after glucose administration. Tissues were collected and fixed in 10% phosphate-buffered formalin for histological analysis. Paraffin-embedded tissues were sectioned (5 μm thick) and stained with hematoxylin and eosin (HE) using standard procedures.

Statistical analysis

For each animal experiment, C57 lean mice and *db/db* mice (n = 3 mice per group) were either untreated (control) or treated with different concentrations of L-81 or 0.005% rosiglitazone (rosig). The experiment was performed 3 times to obtain data with statistical significance. Data shown were obtained from at least 3 independent experiments and presented as mean ± SD. One-way analysis of variance (ANOVA) was used to compare multiple experimental groups. Differences were considered to be statistically significant when $P < 0.05$.

RESULTS

L-81 treatment induced weight loss and ameliorated diabetes

The nonionic surfactant L-81 has been shown to be an effective weight-reducing drug in rats[4,8]. We observed a similar effect in *db/db* mice. Thus, while *db/db* mice weighed 40 g on average at 5-6 wk of age, and their weight continued to increase during observation, mice treated with 1% or 1.5% L-81 for 30 d lost weight to a small extent (n = 3 mice per group) (Figure 1A and B). Their plasma total cholesterol and plasma triglycerol (TG) were also reduced by L-81 treatment (Figure 1C and D). Examination of their epididymal fat pads revealed that adipocytes in mice treated with L-81 were smaller than in the *db/db* controls, although they were still larger than those in C57 lean control mice (Figure 1E). These data showed that L-81 is, as already found in rats, a hypolipidemic agent in *db/db* mice.

While the weight control effect of L-81 is well documented, its implication in diabetes control is less well understood. So we asked, given the fact that L-81 is effective in control of obesity, is it able to ameliorate diabetes? To answer this we measured some diabetic parameters in L-81 treated *db/db* mice. In this study, we compared L-81 with rosig, a peroxisome proliferator-activated receptor gamma (PPAR-γ) agonist and an anti-diabetes drug[17].

Hyperphagia and polydipsia are two common symptoms of diabetes. We first observed whether a 4-wk L-81 treatment could affect the feeding and drinking behaviors of the mice. Compared with lean controls, *db/db* mice ingested about 2 g more food and 4 g more water per day. Inclusion of 1% or 1.5% L-81, or 0.005% of rosig in their diet could reduce their food and water intake to the lean control level (Figure 2A and B).

Fasting plasma glucose levels of the mice were measured at the beginning and the end of a 21 d treatment period. As shown in Figure 2C, *db/db* mice exhibited elevated fasting blood glucose levels of about 25 mmol/L, as compared with 10 mmol/L in lean controls. Twenty one days of rosig or L-81 treatment reduced this parameter to near the lean control level (Figure 2C). *db/db* mice have a typical diabetic blood glucose profile after glucose injection (Figure 2D). Rosig or L-81-treated mice showed improved glucose tolerance, thus their blood glucose level started to drop slightly or stopped increasing at 30 min after injection (Figure 2D). However, the levels were never as low as in lean control mice.

db/db mice are insulin-resistant and therefore have an elevated plasma insulin level[14]. We found that *db/db* mice treated for 21 d with rosig or L-81 exhibited a lowered plasma insulin concentration (Figure 2E). The effects of rosig and L-81 were comparable. Rosig effectively increased the plasma level of adiponectin, an adipocytokine responsible for increasing tissue sensitivity to insulin. This, however, was not observed in L-81 treated mice (Figure 2F). Taken together, oral L-81 treatment can effectively improve diabetic symptoms in the *db/db* mouse model.

L-81 inhibited MTP transcription

L-81 treated cells show decreased activity of MTP. However MTP purified from those cells exhibited normal TG transfer activity[7], suggesting that L-81 does not affect MTP activity post-translationally. We have shown previously that MTP activity is largely controlled at the transcription level[15]. We therefore wished to address the possibility that L-81 controls MTP activity by modulating the transcription of the MTP gene. To do this we transiently transfected a MTP promoter-luciferase reporter construct as previously described[16] into HepG2 and treated the cells with L-81.

As shown in Figure 3A, the expression of luciferase driven by the full-length MTP promoter (-612 to +86) was reduced by about 25% in the presence of 1% L-81. This figure agrees with that observed previously[7]. Given that MTP plays an essential role in the assembly and secretion of apoB-containing lipoproteins, we tested the effect of L-81 on apoB secretion in HepG2 cells. As shown in Figure 3B, L-81 dose-dependently inhibited the apoB secretion in HepG2 cells, while no significant changes were observed in apoA-I secretion. By examining the cell morphology, lactate dehydrogenase activity (a marker of necrosis), and total cellular proteins

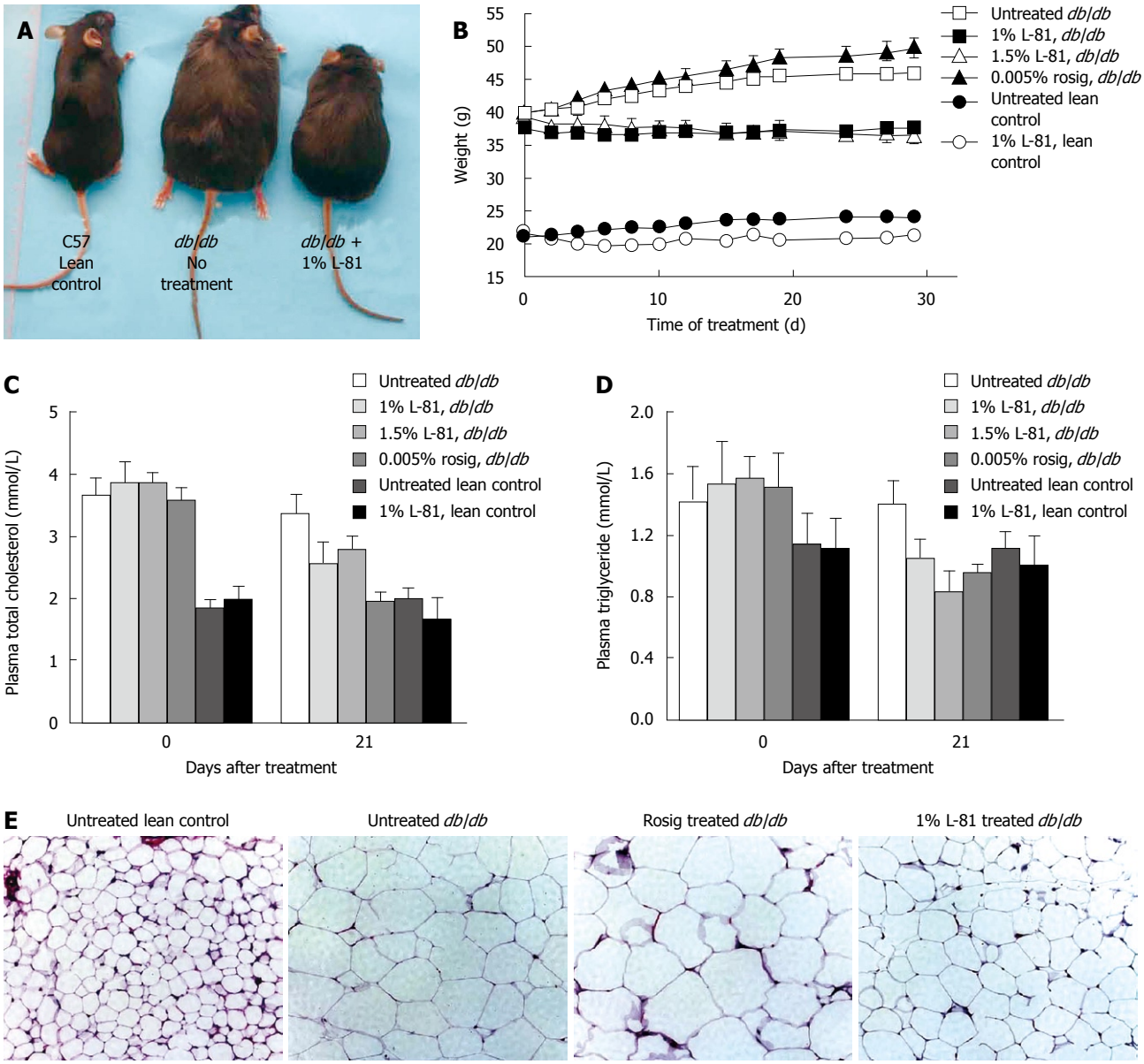

Figure 1 L-81 reduced obesity. A: Body appearances of lean control (left), untreated db/db (middle), and db/db mice fed with chow sprayed with 1% L-81 (right); B: Weight of *db/db* mice or lean control mice receiving different drug treatments over an observation period of 30 d (n = 3 in each group); C: Plasma total cholesterol and (D) plasma triglyceride level of mice treated with the indicated drugs; E: The size of adipocytes in epididymal fat pads of mice treated with the indicated drugs as observed under 100 × magnification.

in HepG2 cells, we also determined that *in vitro* L-81 treatment (0-3 μmol/L) had little or no cytotoxic effect on the cells (data not shown). Taken together, these results demonstrated that L-81 causes a reduction of apoB secretion in HepG2 cells at least partly *via* the inhibition of MTP expression.

To locate the sequence element responsible for mediating the inhibitory effect of L-81 on MTP promoter, we transfected HepG2 cells with various MTP promoter/luciferase reporter deletion constructs (see Figure 3A), and their relative promoter activities were determined from the cells treated with 1% L-81. It was found that the inhibitory effect of L-81 (and insulin) persists up to the deletion construct MTP-115 (Figure 3A), suggesting that L-81 (and insulin) responsive sequence resides between -102 and -115. A mutation of promoter sequence -102 to -108 [where a hepatocyte nuclear factor (HNF)-1 site is located] abolished the inhibitory effect, suggesting that this region is responsible for mediating the inhibition. Either deletion or mutation of the HNF-1 site caused an almost complete shutdown of the MTP promoter activity (Figure 3A), indicating that this site is involved not only in the regulation but also in the basal expression of the MTP gene.

L-81 caused no detectable toxicity to the liver

Pharmacological inhibition of MTP transcription has been explored as a means of treating dyslipidemia. It is believed that by interfering with hepatic and intestinal VLDL packaging, MTP transcription inhibitors would reduce the plasma level of VLDL. Many MTP transcription

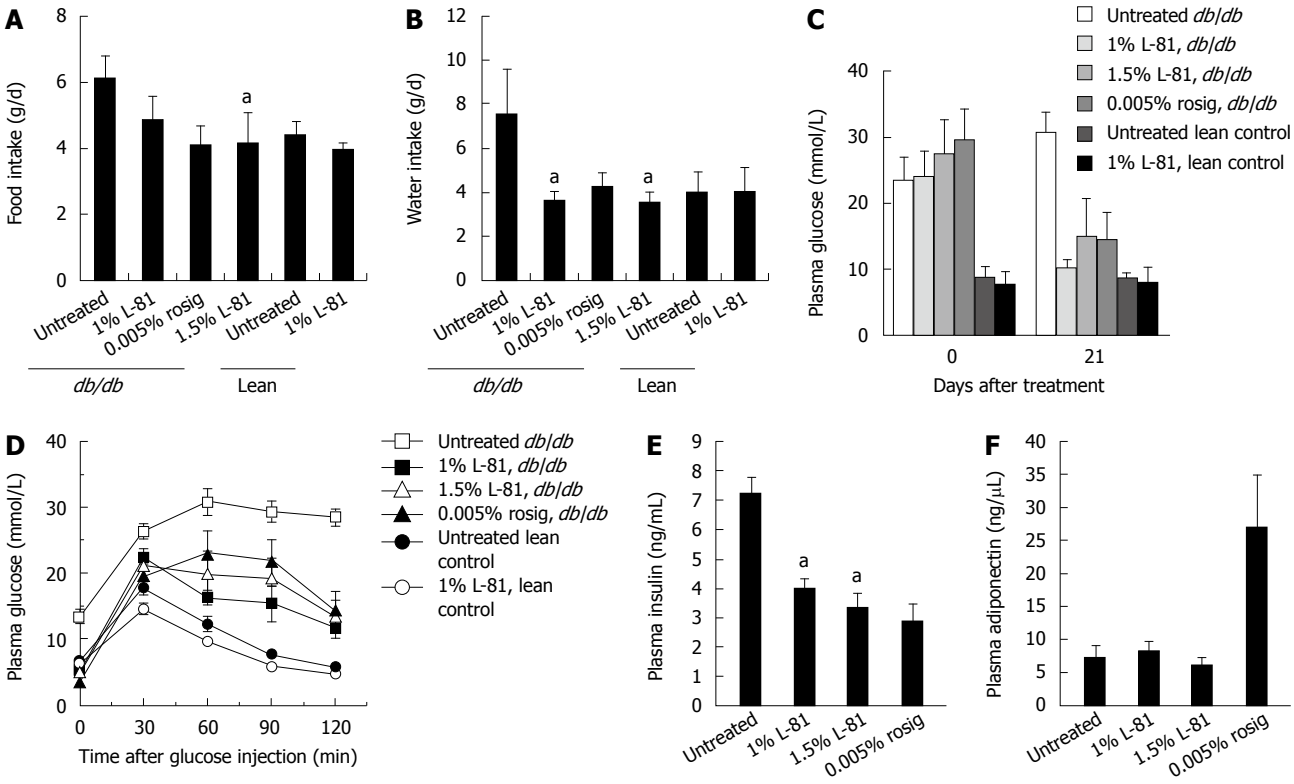

Figure 2 L-81 ameliorated diabetes. A: Food intake and (B) water intake of mice fed on a chow diet, with or without the indicated drugs (n = 3 in each group); C: Fasting plasma glucose levels of lean control mice or *db/db* mice treated with L-81 or rosig before and after a 21 d treatment of the indicated drugs; D: Glucose tolerance of mice treated with the indicated drugs. Mice injected with glucose had their blood glucose level monitored by Onetouch Ultra Blood Glucose Monitoring system; E: Plasma insulin level of *db/db* mice treated with the indicated drugs for 4 wk; F: Plasma adiponectin level of db/db mice treated with the indicated drugs for 4 wk. $^aP < 0.05$ compared with untreated control.

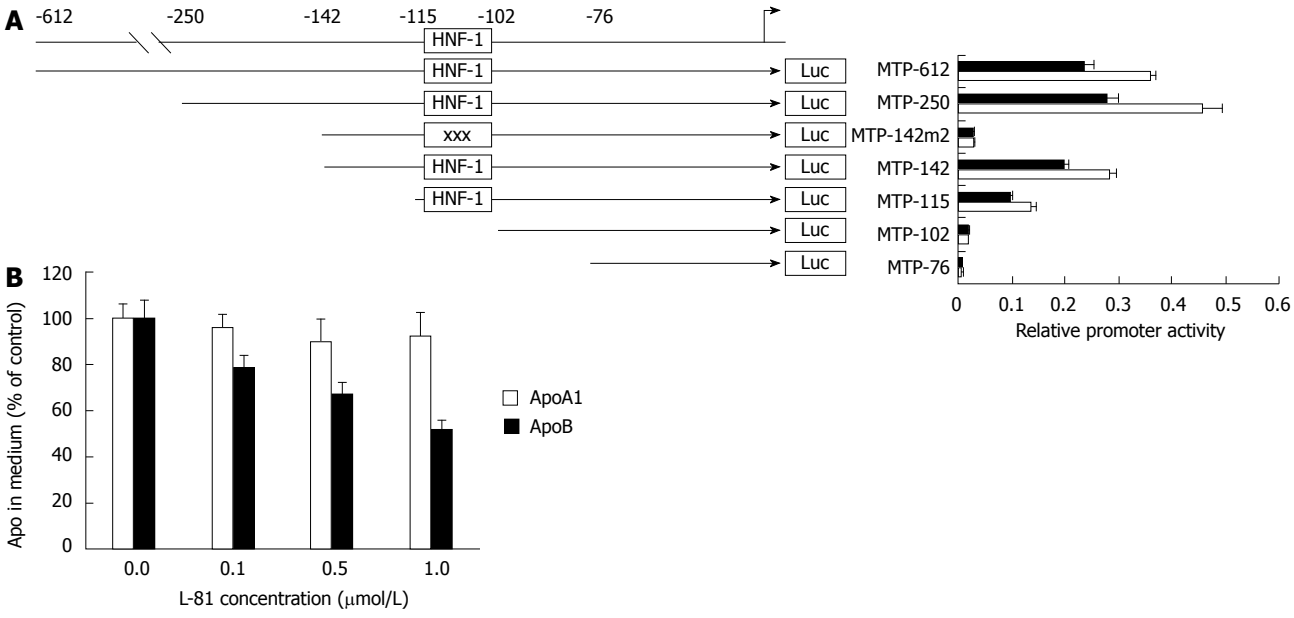

Figure 3 L-81 regulated MTP transcription. A: The human MTP promoter was linked with luciferase reporter. Deletion or mutation constructs of MTP promoter-luciferase were transfected into HepG2 cells. The cells were treated with (black bars) or without (white bars) 1% L-81 in the medium. The luciferase activity in the cell lysate was measured. xxx = disabling mutations; B: L-81 inhibited apoB secretion in HepG2 cells. HepG2 cells were treated with the indicated concentration of L-81. The concentration of apoA-I and apoB in the culture medium were measured and normalized against the untreated control.

inhibitors have been developed and put into clinical trials. However, from the clinical trial data, it is obvious that MTP transcription inhibitors very often cause hepatosteatosis (fatty liver)[18]. We were hence concerned whether L-81's MTP transcription inhibitory effect would lead to hepatosteatosis and a decline of liver function.

To check whether L-81 treatment caused any gross hepatic injury, we measured the plasma ALT level of

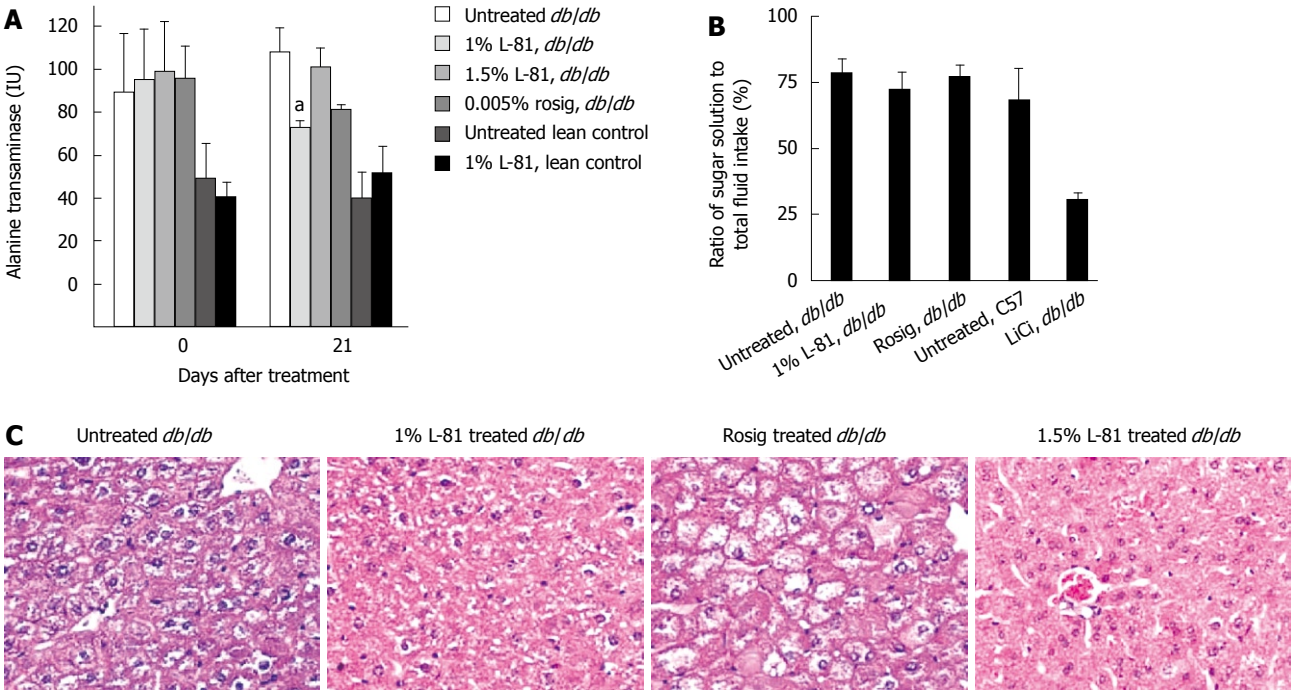

Figure 4 L-81 was not toxic to the liver. A: Plasma ALT level of mice treated with the indicated drugs; B: CTA test of mice treated with the indicated drugs; C: Histological examinations of db/db mice treated with indicated drugs. $^{a}P < 0.05$ compared with untreated *db/db*.

db/db C57L mice after 21 d of L-81 treatment. In lean control mice, the presence of L-81 in the diet did not cause any significant difference in the plasma ALT level, indicating that L-81 did not induce liver injury (Figure 4A). Interestingly, we found that while *db/db* mice generally have a higher level of plasma ALT than C57L lean control mice, L-81 treatment did not result in any significant difference (Figure 4A). Histological examination of the livers of the mice revealed that L-81 treatment caused neither an enlargement of the liver nor any microscopic damage to the liver (Figure 4C).

Conditioned taste aversion (CTA) is a commonly used test to assess whether a particular substance or treatment would render animals ill. L-81 treated mice consumed 72% of their total fluid intake as saccharin in the 2-bottle test, a ratio comparable to that of the untreated *db/db* mice (Figure 4B). Treatment with rosig gave a similar response (Figure 4B). In contrast, administration with lithium chloride produced a robust CTA (Figure 4B). Taken together, we have not observed any toxicity associated with L-81 treatment.

DISCUSSION

The prevalence of obesity is now escalating globally and insulin resistance resulting from the increased fat mass has been identified as a key factor that could drive parallel increases in T2DM[19]. The prevalence of T2DM has reached a pandemic situation in which it is estimated to affect > 6% of the world population. Clinically, patients with T2DM display not only an elevated level of blood glucose, but also an elevated level of plasma lipids. These abnormal levels of circulating lipids are the primary cause of severe macrovascular diseases with poor prognosis, which is responsible for 80% of diabetic mortality and 75% of all hospitalizations for diabetic complications[20]. Indeed, diabetes is recognized as a coronary heart disease "risk equivalent", and the risk of myocardial infarction in diabetic patients is equal to those of non-diabetic patients with known cardiac diseases[21]. Thus, new therapeutic strategies for the treatment of diabetes should not aim only to lower blood glucose level, but also to address diabetes-associated lipid disorders.

Our present study provides evidence for the first time that L-81 is not only a potent lipid-lowering compound but also an anti-diabetic agent in *db/db* mice. L-81 normalizes the levels of plasma lipids and reduces fat mass, and thereby attenuates obesity which is essentially linked to insulin resistance and a number of serious diabetes-associated macrovascular disorders such as coronary, cerebral and peripheral artery diseases[20]. The ability of L-81 to attenuate obesity may play an important role in mediating its antidiabetic effects in *db/db* mice. In fact, the relationship between obesity and insulin resistance/T2DM has been linked in a cause and effect association, as weight loss or gain correlates closely with increasing or decreasing insulin sensitivity, respectively[22-24].

Moreover, L-81 significantly improved fasting glycemic control and glucose tolerance in *db/db* mice. It also reduced hyperphagia and polydipsia, which are symptoms of diabetes mellitus. Remarkably, L-81 caused neither hepatotoxicity nor apparent sickness during the course of treatment. The fact that treatment with L-81 decreased food intake in the *db/db* mice is likely to be an element of the therapeutic action of L-81. Earlier studies showed that L-81 treatment did not affect food

consumption in lean mice[8,25]. Our data on the lean mice are consistent with this previous observation. Compared with rosig, a PPAR-γ agonist[17] and a well marketed anti-diabetic drug, L-81 exhibited comparable potency in lipid and glycemic control without eliciting overt weight gain which is the side effect of the former[26,27].

Consistent with previous studies, we observed that treatment with L-81 led to an accumulation of lipid stored in intestinal villi. Since the turnover rate of the intestinal epithelial lining is rapid, the excess lipids may be excreted together with obsolete cells. It is believed that the intestine is the principal site of action of L-81 when the drug is included in the diet[8,25]. Functionally, L-81 impairs the transepithelial lipid flux during fat absorption and arrests the trafficking of apoB-containing lipoproteins within the enterocytes, which leads to a cytosolic and endoplasmic reticulum lipid accumulation and thereby prevents the absorption of lipids[4,28,29]. This action appears to be selective and does not affect other lipid metabolism such as fatty acid synthesis, cholesterol re-esterification, and more importantly the absorption of other nutrients[4,5,29,30]. Taken together, our present results indicate that L-81 exhibits prominent antidiabetic activities in the diabetic *db/db* mice and suggest that L-81 may represent a new class of anti-diabetic agent conferring both efficacy and safety.

MTP exists in the lumen of the endoplasmic reticulum as a heterodimer with protein-disulfide isomerase and plays a pivotal role in the assembly and secretion of the apoB-containing lipoproteins[31]. Our *in vitro* studies showed that L-81 treatment induced a significant reduction in apoB secretion of HepG2 cells, at least partly *via* the inhibition of MTP gene expression. We also found that the expression of MTP transcripts in the liver of L-81-treated mice was significantly decreased, supporting our *in vitro* data which demonstrated that L-81 could reduce MTP expression. These results indicated that attenuation of the MTP gene expression by L-81 may partially contribute to the improvement of the lipid profiles in diabetic *db/db* mice. Moreover, we showed that L-81 inhibited MTP promoter activity, in a similar fashion as insulin, in L6 cells with an enforced expression of HNF-1α, suggesting that L-81 mimics insulin action and exerts an inhibitory effect on the MTP gene *via* the same HNF-1 element.

We have previously shown that the consensus HNF-1 element is functionally equivalent to the slightly modified HNF-1 element found on the MTP promoter in mediating the negative insulin response[32]. This finding implies that the HNF-1-mediated negative insulin response is not only limited to the MTP gene but also to other genes containing the HNF-1 binding motif. In addition to the MTP gene, the transcription of other HNF-1-containing genes may be elevated under diabetic conditions, which could potentially result in abnormal lipid and glucose metabolism and elevated plasma triglyceride, cholesterol and glucose levels. Thus, compounds like L-81 that can mimic the insulin effect and suppress the transcription of MTP and other HNF-1-containing genes should provide relief to these syndromes. Since HNF-1α contributes to the transcriptional regulation of many rate-limiting steps in gluconeogenesis and also binds to genes whose products are central to normal hepatic functions, including carbohydrate synthesis and storage, lipid metabolism, detoxification, and synthesis of serum proteins[33], the potential regulation of these genes by L-81 warrants further investigation in order to gain more insight into the mechanisms of L-81 action.

COMMENTS

Background

Obesity is common in many developed countries; more than 50% of the adults in the USA are either overweight or obese. The risk of developing type 2 diabetes and cardiovascular diseases increases in parallel with increasing severity of obesity. Therefore, it is crucial to establish effective prevention and treatments for obesity and its associated type 2 diabetes.

Research frontiers

Pluronic L-81 (L-81) effectively inhibits absorption of dietary lipids from the intestine, and secretion of very low density lipoprotein (VLDL) and low density lipoprotein (LDL) from the liver. In this study, the authors demonstrated that oral L-81 treatment can alleviate diabetes symptoms in a mouse model of diabetes (*db/db* mice) and suppress apolipoprotein B (apoB) secretion and microsomal triglyceride protein (MTP) gene transcription in HepG2 cells.

Innovations and breakthroughs

Although L-81 is a potent anti-obesity drug, its potential in alleviating obesity-induced insulin resistance and type 2 diabetes has not been fully explored. This is the first study to report that L-81 has anti-diabetic effects on *db/db* mice, and it mimics the action of insulin by reducing the transcription of the MTP gene.

Applications

By elucidating the molecular mechanism of the anti-diabetic actions of L-81, this study provides the basis for the development of L-81 as a selective insulin mimetic and anti-diabetic agent in the future.

Terminology

Pluronic® surfactants are synthetic copolymers based on ethylene oxide and propylene oxide. They have been widely used in industries as defoaming and antifoaming agents in dishwashing, antifreeze, cutting and grinding fluids. L-81 contains 10% hydrophilic and 90% hydrophobic polyoxyethylene residues with a molecular weight of 2750.

Peer review

The authors investigated the anti-diabetic actions of a nonionic surfactant, L-81, in a mouse model of diabetes. They found that L-81 markedly ameliorated the diabetes symptoms in *db/db* mice by reducing their body weight and plasma glucose, cholesterol and triglyceride levels. Similar to the action of insulin, L-81 was found to inhibit apoB secretion and MTP gene transcription in HepG2 cells. The results are important and lay down the foundation of developing L-81 as an anti-diabetic drug.

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Nanosized As_2O_3/Fe_2O_3 complexes combined with magnetic fluid hyperthermia selectively target liver cancer cells

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Abstract

AIM: To study the methods of preparing the magnetic nano-microspheres of Fe_2O_3 and As_2O_3/Fe_2O_3 complexes and their therapeutic effects with magnetic fluid hyperthermia (MFH).

METHODS: Nanospheres were prepared by chemical co-precipitation and their shape and diameter were observed. Hemolysis, micronucleus, cell viability, and LD_{50} along with other *in vivo* tests were performed to evaluate the Fe_2O_3 microsphere biocompatibility. The inhibition ratio of tumors after Fe_2O_3 and As_2O_3/Fe_2O_3 injections combined with induced hyperthermia in xenograft human hepatocarcinoma was calculated.

RESULTS: Fe_2O_3 and As_2O_3/Fe_2O_3 particles were round with an average diameter of 20 nm and 100 nm as observed under transmission electron microscope. Upon exposure to an alternating magnetic field (AMF), the temperature of the suspension of magnetic particles increased to 41-51℃, depending on different particle concentrations, and remained stable thereafter. Nanosized Fe_2O_3 microspheres are a new kind of biomaterial without cytotoxic effects. The LD_{50} of both Fe_2O_3 and As_2O_3/Fe_2O_3 in mice was higher than 5 g/kg. One to four weeks after Fe_2O_3 and As_2O_3/Fe_2O_3 complex injections into healthy pig livers, no significant differences were found in serum AST, ALT, BUN and Cr levels among the pigs of all groups ($P > 0.05$), and no obvious pathological alterations were observed. After exposure to alternating magnetic fields, the inhibition ratio of the tumors was significantly different from controls in the Fe_2O_3 and As_2O_3/Fe_2O_3 groups (68.74% and 82.79%, respectively; $P < 0.01$). Tumors of mice in treatment groups showed obvious necrosis, while normal tissues adjoining the tumor and internal organs did not.

CONCLUSION: Fe_2O_3 and As_2O_3/Fe_2O_3 complexes exerted radiofrequency-induced hyperthermia and drug toxicity on tumors without any liver or kidney damage. Therefore, nanospheres are ideal carriers for tumor-targeted therapy.

Key words: Liver cancer; Magnetic fluid hyperthermia; Nanoparticle; As_2O_3

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors in China, and the incidence has increased in recent years. Current therapeutic options remain unsatisfactory for most patients. Surgical resection has been recognized as the most effective method for the treatment of hepatocarcinoma, but it is only indicated for a small number of hepatocarcinoma patients[1,2]. Therefore, it is crucial to identify a new method to treat hepatocarcinoma.

In recent years, radiofrequency-induced hyperthermia has increasingly attracted attention for the generation of heat in a desired zone, even in tumors deeply located inside a patient's body. During exposure to alternating

magnetic field (AMF), magnetic particles can absorb energy and transform it into heat at temperatures of 42-45℃, at which tumor cells are very sensitive[3]. In addition, the use of magnetic nanospheres, which can carry the magnetic particles into the tumor cells very easily, can greatly enhance the effects of the thermotherapy. In our research, we attempted to prepare a kind of new magnetic material that contained As_2O_3. We transformed the energy of the radio waves into heat to kill tumor cells and explored the therapeutic effects of nanospheres for the treatment of hepatocarcinoma.

MATERIALS AND METHODS

Materials

As_2O_3 and dimethyl sulfoxide (DMSO) was purchased from Sigma. RPMI-1640 medium was obtained from GIBCOL-BRL. Newborn calf serum was from Si-Ji-Qing Biotechnology Co. (China). HEPES, Trypsin and methyl thiazolyl tetrazolium (MTT) were purchased from AMRESCO. The transmission electron microscope (TEM) used was a H-600 model (Hitachi, Japan) and the scanning electron microscope (SEM) model was JEOL JSM-6360LV (Japan). The energy dispersive spectrometer (EDS) was purchased from Thermo NORAN Vantage (USA).

L929 cells (human fibroblast cell line) and SMMC-7721 cells (human liver cancer cell line) were purchased from the Institute of Biochemistry and Cell Biology, Shanghai Institute of Biological Sciences, Chinese Academy of Sciences.

BALB/C nude mice (male, 10-wk-old) were purchased from the Lakes Animal Experimental Center of the Institute of Biochemistry and Cell Biology, Shanghai Institute of Biological Sciences, Chinese Academy of Sciences.

Methods

Preparation and characteristics of Fe_2O_3 and As_2O_3/Fe_2O_3 nanoparticles: Fe_2O_3 magnetic nanoparticles were prepared according to the method previously described[4]. Fe_2O_3 magnetic nanoparticles were added into a solution of As_2O_3 (0.01 mg/mL, pH = 5, adjusted by acetic acid) under a condition of supersonic dispersion. After 30 min at 80℃, the products were centrifuged at 2000 r/min for 10 min, rinsed twice by absolute alcohol, and then dried in a vacuum. The diameter and composition of Fe_2O_3 and As_2O_3/Fe_2O_3 were examined under TEM and EDS.

A heating test was performed to detect the thermodynamic characteristics of the magnetic particles. Various doses of Fe_2O_3 and As_2O_3/Fe_2O_3 particles were decentralized in 0.9% NaCl. The concentrations of Fe_2O_3 were 2, 4, 6 and 8 g/L. Two milliliters of nanoparticle fluid was then added to a flat-bottomed cuvette to reach a level of 5 mm from the bottom of the cuvette and in the center of the hyperthermia-coil of a high frequency electromagnetic field. The output electric current was 300 A, and the fluid was heated for 1 h with temperature measurement at 5 min intervals.

Biocompatibility study of Fe_2O_3 nanoparticles: MTT assay, hemolytic test, and micronucleus assay were performed to test the *in vitro* cytotoxicity of Fe_2O_3 nanoparticles. To perform the MTT assay, L929 cells were cultured in RPMI-1640 media supplemented with 10% heat-inactivated calf serum, penicillin (100 U/mL) and streptomycin (100 mg/mL) and grown in the presence of 5% CO_2 at 37℃. Cells were seeded in a 96-well plate and treated with 200 μL Fe_2O_3 nanoparticle fluid at various concentrations (100%, 75%, 50% and 25%) for 48 h and with 5 μmol/L of As_2O_3 as a positive control. Subsequently, 20 μL (5 g/L) MTT was added to the cells in each well and incubated for 4 h at 37℃. Culture media was discarded and 150 μL of DMSO was added and subjected to vibration for 10 min. The absorbance (A) value was measured at a wavelength of 493 nm. The cell relative growth rate (RGR) was calculated as follows: (A of experimental group/A of control group) × 100%.

For the hemolytic test, 50 mL of Fe_2O_3 and As_2O_3/Fe_2O_3 was centrifuged at 2000 r/min for 10 min 3 times, then suspended and incubated at 37℃, and after 30 min a liquid-extract was obtained. Ten milliliters of 0.9% NaCl and 10 mL of double distilled water were used as negative (0% hemolysis) and positive (100% hemolysis) controls, respectively. Each group contained three tubes. Diluted anticoagulated rabbit blood (0.2 mL) was added to each tube, which had been pre-heated at 37℃ for 30 min. Contents of all the tubes were incubated in a water bath at 37℃ for 60 min. All tubes were centrifuged at 2500 r/min for 5 min and the supernatant was taken to estimate free hemoglobin. Absorbance was measured and recorded at 540 nm. In general, the optical density was 0.8 ± 0.3 in positive control groups and was no more than 0.03 in negative control groups. The hemolysis rate (HR) was calculated as follows: HR (%) = (A of experimental group - A of negative control group)/(A of positive control group - A of negative control group) × 100%.

For the micronucleus assay, 60 mice were randomly divided into six groups, with five females and five males in each group. Animals were injected intraperitoneally with 100 g/L of Fe_2O_3 or As_2O_3/Fe_2O_3 (40 mg/kg) twice at a 24 h interval. The negative group (with 0.9% NaCl) and positive group (with CT × 40 mg/kg) were set as control groups. Six hours after the second injection, all the mice were killed. The thighbone marrows were extracted for smears, methanol-fixed for 5 min, then dyed with Giemsa for 15 min. For each smear, 1000 polychromatic erythrocytes (PEC) were counted, and the number of PEC containing micronucleus was calculated (MN). Poisson distribution verified the statistical difference of each group.

We also studied the *in vivo* histotoxicity of nanoparticles. The Kun Ming mice were divided into 15 groups randomly with five females and five males in each group. Various amounts of 100 g/L Fe_2O_3 and As_2O_3/Fe_2O_3 nanoparticles were intraperitoneally injected into each mouse of seven groups at 1.25, 1.75, 2.5, 3.5, 5,

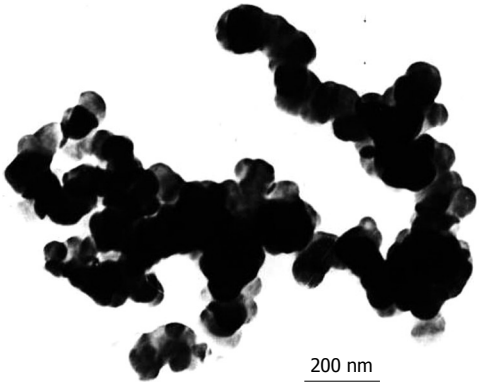

Figure 1 Shape of As_2O_3/Fe_2O_3 observed under TEM.

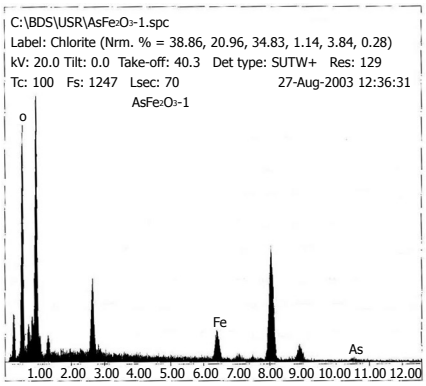

Figure 2 EDS results of As_2O_3/Fe_2O_3.

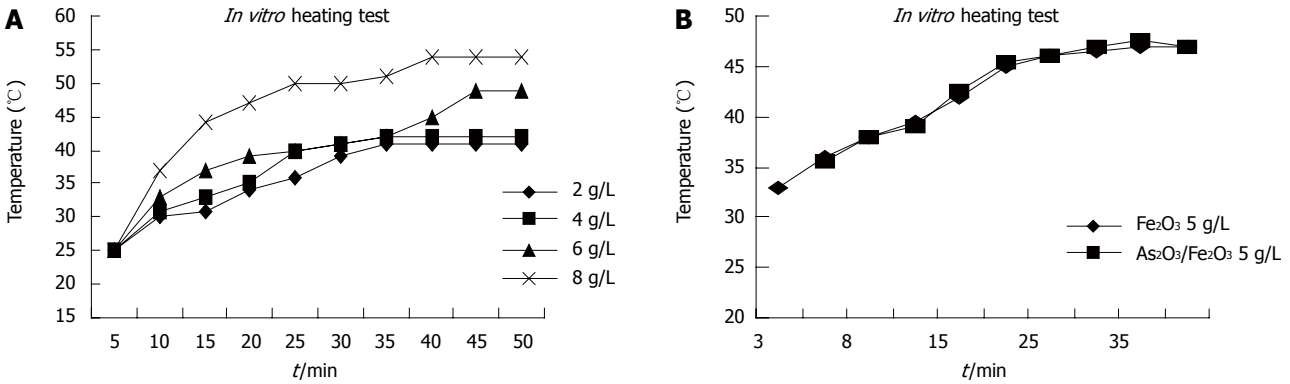

Figure 3 Thermodynamic test. A: Various doses of Fe_2O_3; B: The concentration of Fe_2O_3 is 5 g/L.

7.07 and 10 g/kg according to their weight. The negative control group was injected with the same volume of 0.9% NaCl, and the mice were observed in the following 15 d. The median lethal dose (LD_{50}) was evaluated by the Karber method.

Sixteen healthy pigs were divided randomly into four groups (control group, Fe_2O_3 low dose, Fe_2O_3 high dose, and the As_2O_3/Fe_2O_3 group). Fe_2O_3 or As_2O_3/Fe_2O_3 (10 g/L) was injected into the liver of pigs in the experimental groups. Four pigs from each group were killed from 1 to 4 wk after injection. Serum AST, ALT, BUN and Cr were measured. Livers were harvested and dissected into 1 mm^3 specimens. Subsequently, the samples were fixed in 4% glutaraldehyde and were prepared into ultrathin sections (60 nm) to be examined under TEM and EDS.

Therapeutic effect of As_2O_3/Fe_2O_3 in combination with MFH on xenograft liver cancer

Inhibition of SMMC-7721 cell proliferation was measured by MTT assay according to the method described above. Xenograft tumors were induced in the subcutaneous tissue around the right shoulder of nude mice with SMMC-7721 cells. Once the tumor diameter increased to 0.2-0.4 cm, mice were divided into 6 groups: (1) the control (sterile 0.9% NaCl); (2) As_2O_3 (5 μmol/L As_2O_3); (3) Fe_2O_3 (5 g/L Fe_2O_3); (4) As_2O_3/Fe_2O_3 (5 g/L Fe_2O_3); (5) $Fe_2O_3$with hyperthermia; and (6) As_2O_3/Fe_2O_3 with hyperthermia. Each group contained eight mice. They were injected into the tumors at 1/2 of the volume of the tumor. The tumors of the mice in groups 5 and 6 were exposed to a high-frequency alternating magnetic field and irradiated for 30 min. The treatment was given three times at 24 h intervals. After 45 d, all the mice were killed. The weight and volume inhibitory rates of the tumor were calculated as follows: IW = (1 - the weight of tumor of experimental group/ the weight of tumor of control group) ×100%; Iv = (1 - the volume of tumor of experimental group/the volume of tumor of control group).

Statistical analysis

Values were expressed as mean ± SD. The data were analyzed with SPSS 11.5 and SAS 10.0 software packages. Differences in the results were considered statistically significant when $P < 0.05$.

RESULTS

Characteristics of Fe_2O_3 and As_2O_3/Fe_2O_3 nanoparticles

Under TEM, the nanospheres appeared to be roughly spherical, brown particles that could be suspended stably in water with good dispersibility. The diameter of Fe_2O_3 particles was about 20 nm, and the diameter of As_2O_3/Fe_2O_3 particles was about 100 nm as shown in Figure 1. The EDS result verified that the nanoparticles contained magnetic particles and As_2O_3 (Figure 2).

Figure 3A shows the thermodynamic tests of various

Table 1 Results of MTT test (mean ± SD)

Group	Absorbance value	RGR (%)	Cytotoxicity gradations
Negative control	0.4671 ± 0.0103	100.00	0
25% Fe_2O_3	0.4793 ± 0.0210	102.63	0
50% Fe_2O_3	0.4501 ± 0.0101	96.39	0
100% Fe_2O_3	0.4453 ± 0.0108	95.35	0
25% As_2O_3/Fe_2O_3	0.4373 ± 0.0210	93.64	0
50% As_2O_3/Fe_2O_3	0.1788 ± 0.0247	38.29	3
100% As_2O_3/Fe_2O_3	0.1273 ± 0.0073	27.26	3
As_2O_3 (5 μmol/L)	0.1322 ± 0.0090	30.12	3
Positive control	0.0733 ± 0.0050	15.70	4

Table 3 Results of micronucleus assay (n = 10)

Groups	PEC	PEC containing MN	MN-formation rates (%), mean ± SD
Negative control	10000	24	0.24 ± 1.58
Positive control	10000	241	24.1 ± 4.63
Fe_2O_3	10000	18[a]	0.28 ± 1.40
As_2O_3/Fe_2O_3	10000	29[a]	0.26 ± 1.65

[a]P < 0.05 compared with negative control group.

Table 5 Results of acute toxicity test of As_2O_3/Fe_2O_3

Groups	Dose (g/kg)	Log	N	Deaths (N)	Mortality % (p)	Survival % (q)	p × q
1	10.00	1.000	10	10	100	0	0.00
2	7.07	0.849	10	5	50	50	0.25
3	5.00	0.699	10	3	30	70	0.21
4	3.50	0.544	10	3	30	70	0.21
5	2.50	0.398	10	1	10	90	0.09
6	1.75	0.243	10	0	0	100	0.00
		i = 0.15				∑p = 2.2	

$lgLD_{50}$ = 1-0.15 (2.2-0.5) = 0.745, Sm = 0.0414, As_2O_3/Fe_2O_3 Lg LD_{50} and its 95% CI: 0.745 ± 1.96 × 0.0414 = 0.745 ± 0.0811, As_2O_3/Fe_2O_3 LD_{50} and its 95% CI: 5.56 g/kg (4.56-6.70 g/kg).

doses of magnetic nanoparticles. Fe_2O_3 particles were decentralized in 0.9% NaCl and exposed to a high-frequency alternating electromagnetic field (output current equal to 300 A) for 60 min. The temperature rose rapidly within 5 min and slowly continued to increase from 5-40 min, and remained stable after 40 min. The temperature of the magnetic fluid (MF) rose from 41℃ to 51℃, depending on the different concentrations. The results showed that Fe_2O_3 nanoparticles had good power absorption capabilities in the high-frequency alternating electromagnetic field, and had strong magnetic responsiveness. We selected a suitable temperature range (42-46℃) for tumor hyperthermia[5] by adjusting the concentration of Fe_2O_3. We chose a concentration of 5 g/L for Fe_2O_3, and at this concentration, the temperature rose to 46℃ in MFH (Figure 3B).

Biocompatibility study of Fe_2O_3 and As_2O_3/Fe_2O_3 nanoparticles

MTT assay: The RGR of L929 cells treated with 25%, 50%, and 100% of liquid-extract of Fe_2O_3 were 102.63%, 96.39%, and 95.35%; for As_2O_3/Fe_2O_3 were 93.64%, 38.29%, and 27.26%, respectively. The value of the As_2O_3 group (5 μmol/L) was 30.12% (Table 1). The results corresponded to the cellular morphological changes observed under an inverted microscope.

Table 2 Results of hemolytic test of Fe_2O_3 and As_2O_3/Fe_2O_3 liquid extracts

Group	Absorbance (A)			Average	Hemolysis
	1	2	3	A	rate (HR, %)
Negative control	0.234	0.234	0.236	0.235	
Fe_2O_3 extract	0.232	0.235	0.233	0.233	0.00
As_2O_3/Fe_2O_3 extract	0.246	0.246	0.246	0.246	0.77
Positive control	1.688	1.776	1.521	1.662	

Table 4 Results of acute toxicity test of Fe_2O_3

Groups	Dose (g/kg)	Log	N	Deaths (N)	Mortality % (p)	Survival % (q)	p × q
1	10.00	1.000	10	10	100	0	0.00
2	7.07	0.849	10	4	40	60	0.24
3	5.00	0.699	10	4	40	60	0.24
4	3.50	0.544	10	2	20	80	0.16
5	2.50	0.398	10	0	0	100	0.00
6	1.75	0.243	10	1	10	90	0.09
7	1.25	0.097	10	0	0	100	0.00
		i = 0.15				∑p = 2.1	

$lgLD_{50}$ = 1-0.15 (2.1-0.5) = 0.76, Sm = 0.04, Fe_2O_3 Lg LD_{50} and its 95% CI: 0.76 ± 1.96 × 0.04 = 0.76 ± 0.079, Fe_2O_3 LD_{50} and its 95% CI: 5.75 g/kg (4.80-6.90 g/kg).

Hemolytic test: The Absorbance of each group was observed at 545 nm. As shown in Table 2, the HR of Fe_2O_3 and As_2O_3/Fe_2O_3 nanoparticles was 0% and 0.77%, which is far less than the standard 5% that indicates a hemolytic reaction.

Micronucleus assay: The MN formation rates of Fe_2O_3, As_2O_3/Fe_2O_3, the negative control and the positive control groups were 0.28%, 0.26%, 0.24%, and 24.1%, respectively (Table 3).

Median lethal dose (LD_{50}) determination: The mice receiving various doses were observed over the subsequent 15 d and the experimental animals died in succession. The LD_{50} was evaluated by the Karber method. The LD_{50} of mice receiving Fe_2O_3 and As_2O_3/Fe_2O_3 were 5.75 g/kg and 5.56 g/kg, respectively (Tables 4 and 5).

Biocompatibility study in pigs: One to four weeks after injection of Fe_2O_3 and As_2O_3/Fe_2O_3 into healthy pig livers, no significant differences were found in serum AST, ALT, BUN and Cr levels among pigs of all groups (P > 0.05), and no obvious pathological alterations were observed (Table 6). EDS examination revealed that in the As_2O_3/Fe_2O_3 complex group, numerous black

Table 6 Results of biocompatibility study *in vivo* (mean ± SD)

Groups	TB	ALT	AST	Bun	Cr
Control	4.8 ± 1.67	41.5 ± 10.08	92.25 ± 46.81	3.3 ± 0.36	86.5 ± 6.19
Fe_2O_3 low dose	8.025 ± 2.70	44.25 ± 16.35	87 ± 29.01	3 ± 0.99	83.25 ± 4.65
Fe_2O_3 high dose	6.45 ± 2.97	34.5 ± 9.61	71.25 ± 35.64	2.475 ± 0.46	77.5 ± 15.44
As_2O_3/Fe_2O_3	7.93 ± 2.66	47.25 ± 14.19	52.75 ± 5.5	2.5 ± 0.25	92.25 ± 19.69

Compared with control group: $P > 0.05$.

Table 7 Volume and mass inhibitory rate of xenograft liver cancer in nude mice after treatment

Groups	Tumor mass (g, mean ± SD)	Mass inhibitory rate (%)	Tumor volume (cm^3, mean ± SD)	Volume inhibitory rate (%)
Control	0.6145 ± 0.2296	0.00	0.7195 ± 0.3231	0.00
As_2O_3	0.5885 ± 0.1628	5.86	0.6015 ± 0.2282	16.40
Fe_2O_3	0.5365 ± 0.2792	12.69	0.5997 ± 0.2518	16.65
As_2O_3/Fe_2O_3	0.4548 ± 0.2591	26.04	0.6252 ± 0.4034	21.45
Fe_2O_3 with MFH	0.1921 ± 0.0395	68.74[b]	0.2373 ± 0.0874	67.02[b]
As_2O_3/Fe_2O_3 with MFH	0.1057 ± 0.0510	82.79[b,c]	0.1183 ± 0.0726	83.56[b,c]

[b]$P < 0.01$ *vs* control group; [c]$P < 0.05$ *vs* Fe_2O_3 with MFH group.

nanosized As_2O_3/Fe_2O_3 complexes had accumulated in the liver tissue of pigs.

Therapeutic effect of Fe_2O_3 and As_2O_3/Fe_2O_3

Morphological changes of apoptotic SMMC-7721 cells: The morphological changes of SMMC-7721 cells after treatment were observed under inverted microscopy. As shown in Figure 4, cells in the control and the Fe_2O_3 groups exhibited a normal shape, clear edge, and no cell fragmentation (Figure 4A). In the groups of As_2O_3, the As_2O_3/Fe_2O_3, the Fe_2O_3 and As_2O_3/Fe_2O_3 combined with MFH, the SMMC-7721 cells became small and global. Some shrunk and even a portion of the cells were suspended, which revealed the typical changes associated with apoptosis (Figure 4B-D). The results showed that both As_2O_3 (5 μmol/L) and MFH (at 46℃) could damage liver cells by inducing apoptosis.

Inhibition of SMMC-7721 cell proliferation after treatment with Fe_2O_3 and As_2O_3/Fe_2O_3 combined with MFH: The results of MTT assay are shown in Figure 5. The cell survival rates of cells treated with Fe_2O_3 and As_2O_3/Fe_2O_3 combined with MFH were 19.66% and 19.95%, respectively, which was statistically different from the negative group ($P < 0.01$). So, the therapeutic effect of nanosized As_2O_3/Fe_2O_3 complexes in combination with MFH on SMMC-7712 cells is much better than that of As_2O_3 or Fe_2O_3 nanoparticles alone.

***In vivo* inhibitory effect of Fe_2O_3 and As_2O_3/Fe_2O_3 combined with MFH on xenograft liver cancer in nude mice:** Animal experiments showed that tumors in the experimental groups became smaller (Figure 6). The mass and volume inhibitory ratio of the As_2O_3/Fe_2O_3 combined with MFH were IM = 82.79% and IV = 83.56%, respectively, which was much higher than that of the other groups (Table 7). Compared with control and experimental groups, each group was markedly different from the controls ($P < 0.01$). Histological examination in the As_2O_3/Fe_2O_3 group revealed that there was an accumulation of black nanosized As_2O_3/Fe_2O_3 particles at the stroma in the margin of the tumors. Many of the tumor cells disappeared at the site adjacent to this accumulation, and a necrotic zone was found surrounding the material (Figure 6).

DISCUSSION

As_2O_3, a traditional Chinese medicine, plays an important role in the treatment and research for human cancers such as acute promyelocytic leukemia (APL), myeloid leukemia, gastric cancer, breast cancer, neuroblastoma and esophageal carcinoma, as well as head and neck cancers[6-8], however, there are many limitations to its use due to its form. Patients treated with As_2O_3 suffered from acute and chronic side effects such as gastrointestinal reactions, which are often severe or fatal[9-11]. Moreover, it has been generally considered to be an extremely effective environmental cocarcinogen for some human malignancies, especially for skin and lung cancer[12]. Therefore, enhancing the curative effect and reducing the toxicity of As_2O_3 by changing its form is of great importance.

Hyperthermia for tumor therapy has been a long-standing modality. At present, thermotherapy is commonly used clinically with such applications as radiofrequency, microwave and lasers, all of which have many limitations for tumor hyperthermia. In 1997, Jordan[13] discovered that a nanoscaled magnetic fluid could be absorbed with much higher power in an alternating magnetic field, and used to treat disease or tumors. This treatment is named "Magnetic Fluid Hyperthermia (MFH)". MFH has a high ability to target and localize thermogenic actions. Therefore,

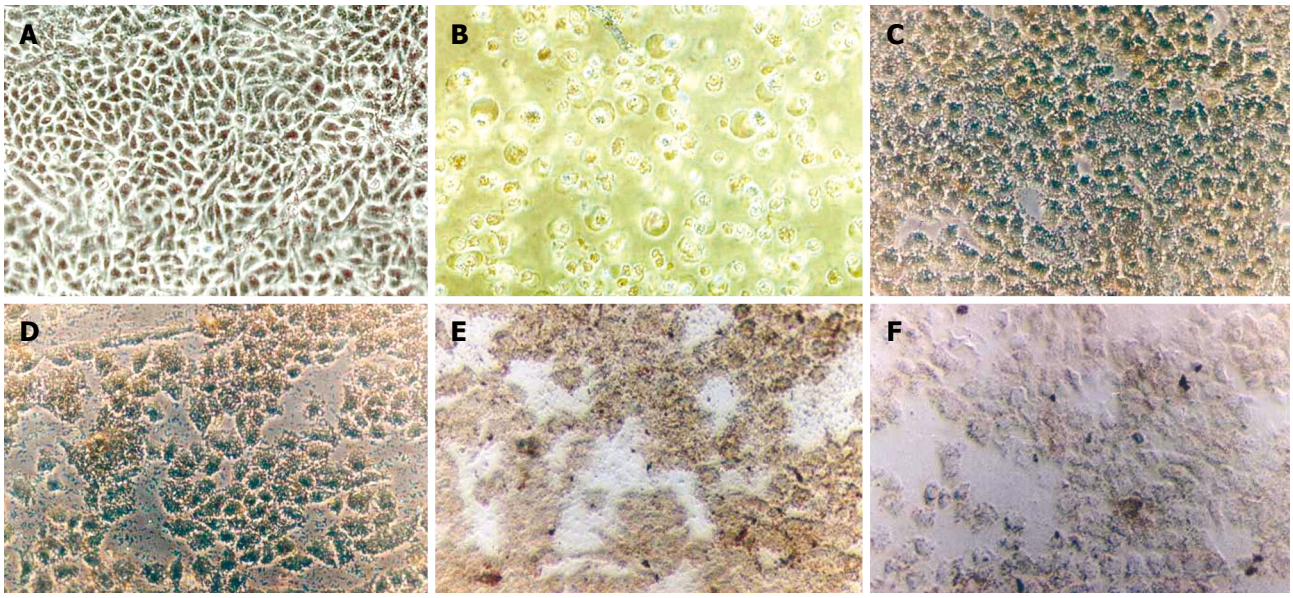

Figure 4 Inverted microscopy of SMMC-7721 cells treated by different methods. A: Negative control group; B: As_2O_3 (5 μmol/L) group; C: Fe_2O_3; D: As_2O_3/Fe_2O_3 group; E: Fe_2O_3 combined with MFH group; F: As_2O_3/Fe_2O_3 combined with MFH group (× 200).

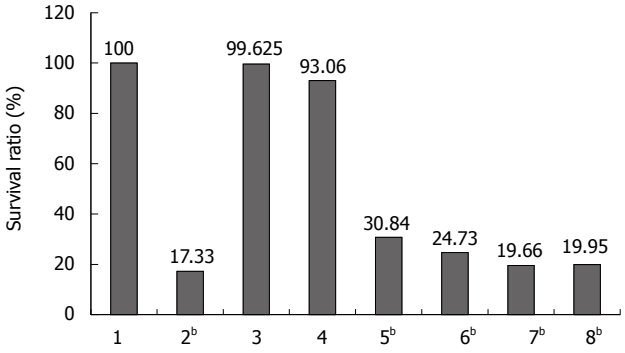

Figure 5 Results of MTT assay of SMMC-7721 cells treated with Fe_2O_3 and As_2O_3/Fe_2O_3 combined with MFH. 1: Negative control; 2: Positive control; 3: MFH alone; 4: Fe_2O_3 (5 g/L); 5: As_2O_3/Fe_2O_3 (5 g/L); 6: As_2O_3 (5 μmol/L); 7: Fe_2O_3 (5 g/L) combined with MFH; 8: As_2O_3/Fe_2O_3 (5 g/L) combined with MFH. ($^bP < 0.01$ *vs* negative group).

Figure 6 Morphological changes of tumors from tumor-bearing nude mice treated by various methods. 1: Control; 2: As_2O_3; 3: Fe_2O_3; 4: As_2O_3/Fe_2O_3; 5: Fe_2O_3 combined with MFH; 6: As_2O_3/Fe_2O_3 combined with MFH.

tissue without magnetic particles would not be damaged.

Nanoparticles combined with MFH may be a potential method to treat tumors. In our study, As_2O_3/Fe_2O_3 complexes were prepared as a new magnetic material. Observed under TEM, they are round or elliptical, disperse well and are about 100 nm in diameter. In addition, they have good power absorption capabilities in a high-frequency alternating electromagnetic field. The temperature can rapidly reach 46℃ within 5 min, which can kill tumor cells while having little effect on normal cells.

However, the biomaterials would be in direct contact with tissues and cells when introduced into the body, so their biocompatibility had to be evaluated before they could be applied in a clinic setting. Many studies have shown that some materials show signs of toxicity when their diameters are reduced to nanoscale[14]. Therefore, the potential hazards and bio-safety of Fe_2O_3 microspheres should be particularly observed when they are applied to tissues. Biomaterials must not only have long-term stability in biotic conditions, but also have no harmful effects on tissues, blood or the immune system. In our work, referring to ISO10993-1992 and other international standards[15-17], we evaluated the nanoparticles using an *in vitro* cytotoxicity test, hemolytic test, a micronucleus experiment, by calculating the LD_{50}, and an *in vivo* study. MTT results showed that Fe_2O_3 nanoparticles had no significant effect on cellular proliferation when treated with various doses of extracted liquids of Fe_2O_3 nanoparticles. The cytotoxicities were 0 grade (RGR > 75%) indicating that there was no evidence of cytotoxicity. The results of the hemolytic test demonstrated that the hemolytic rate of the liquid-extracts of Fe_2O_3 and As_2O_3/Fe_2O_3 were 0.% and 0.77%, far less than 5%. This finding indicated that Fe_2O_3 had no hemolytic reaction when in direct contact with blood and was consistent with the requirement of hemolytic tests for biomaterials. Genotoxicity and

carcinogenicity tests answer the most complicated questions about biomaterials. The micronucleus assay is a rapid detection method to evaluate whether a biomaterial would damage chromosomes or interfere with cellular mitosis. This method can rapidly monitor acute and/or chronic genotoxicity, and does not require cultured cells[18]. In our study, we compared Fe_2O_3 groups with the negative control group and found no significant difference ($P > 0.05$) in the micronucleus formation rate. However, when we compared these groups with the positive control group, the result was significantly different ($P < 0.05$).

Therefore, Fe_2O_3 nanoparticles were not carcinogenic or mutagenic. However, the results of the acute toxicity test revealed that Fe_2O_3 nanoparticles intraperitoneally injected into the mice had low toxicity. The LD_{50} was equal to 5 g/kg, which is in the "no toxicity" category according to the standard of acute toxicity gradation of WHO. The LD_{50} of Fe_2O_3 for the mice was 5.75 g/kg with a 95% confidence interval of 4.8-6.9 g/kg. So, Fe_2O_3 also belonged to the "no toxicity" category and had a wide safety value margin. When we injected Fe_2O_3 into livers of healthy pigs, no significant differences in serum AST, ALT, BUN and Cr levels were found among pigs of all groups ($P > 0.05$), and no obvious pathological alterations were observed. From the results of our experiment, we believe that Fe_2O_3 demonstrated no toxic effects, is a highly biocompatible material and may be suitable for further applications in tumor hyperthermia.

We studied the therapeutic effect of Fe_2O_3 and As_2O_3/Fe_2O_3 combined with MFH on liver cancer *in vitro* and i*n vivo*. We injected Fe_2O_3 and As_2O_3/Fe_2O_3 into the tumor tissues instead of the normal tissue boundary of the tumor. Thus, the nanoparticles were delivered into the desired zone. This method allows thermogenic action to be administered locally, even in tumors located deep inside bodies, while minimizing heating of normal tissue around the tumor[19,20]. Compared with As_2O_3/Fe_2O_3 groups, As_2O_3/Fe_2O_3 combined with MFH had a better inhibitory effect on xenograft liver tumors, which indicates that MFH had a significant therapeutic effect. Much to our surprise, As_2O_3/Fe_2O_3 combined with MFH was the best therapeutic agent among all the groups tested. This result revealed that As_2O_3/Fe_2O_3 combined with MFH had two functions: chemotherapy of As_2O_3 and thermotherapy of magnetic Fe_2O_3 nanoparticles.

In conclusion, As_2O_3/Fe_2O_3 combined with MFH is a new biomaterial with low toxicity. However, we must acknowledge that our studies have limitations, and more researches should be carried out in the future. Although there is still a long way to go before the technology can be applied to clinical treatment, this method may develop into a new approach for the treatment of liver cancer and other solid tumors.

COMMENTS

Background

Hepatocellular carcinoma is one of the most common malignant tumors in China, and has a low recovery rate, so it is necessary to search for a new method to treat liver tumors.

Innovations and breakthroughs

Nanoparticles combined with magnetic fluid hyperthermia (MFH) have become a potential method to treat tumors. In this study, As_2O_3/Fe_2O_3 complexes were found to have two functions, chemotherapy of As_2O_3, and thermotherapy of magnetic Fe_2O_3 nanoparticles.

Applications

As_2O_3/Fe_2O_3 combined with MFH is a new biomaterial with good therapeutic effects on liver cancer. This preparation may be developed into a new agent for the treatment of liver cancer and other solid tumors.

Terminology

MFH is a method that can target and localize thermogenic actions. Therefore, the tissue surrounding the tumor without magnetic particles would not be damaged.

Peer review

The manuscript describes the therapeutic potential of nanosized As_2O_3/Fe_2O_3 complexes in combination with MFH on liver cancer cells. The authors analyzed the toxicity and therapeutic potentials of various concentrations of Fe_2O_3 and As_2O_3/Fe_2O_3. Interestingly, they found significant antitumor effects of those compounds against xenograft tumors of liver cancer cells when combined with magnetic fluid hyperthermia. The data are important and promising.

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ORIGINAL ARTICLES

Clinicopathological analysis of paraganglioma with literature review

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Abstract

AIM: To investigate the 152 cases of paragangliomas resected over the past 32 years in West China Hospital clinicopathologically.

METHODS: All cases of paragangliomas diagnosed at the Department of Gastrointestinal Surgery and Department of Pathology, West China Hospital, China were reviewed. The pathological documents were supplied by the Department of Pathology, West China Hospital, and other necessary data were extracted from the hospital records. The statistical analyses were performed by survival analysis (Kaplan-Meier method), descriptive statistical analyses and χ^2 analysis.

RESULTS: The neuroendocrine marker vimentin was found to be selectively expressed in the benign tumors, and there were significant differences in the expression of those markers in both benign and malignant tumors. The survival analysis revealed that survival correlated significantly with the malignancy, metastasis and nodal status.

CONCLUSION: Vimentin may be useful in the differential diagnosis between malignant and benign tumors. The difference in the expression of this marker in the tumors could be a clue to the future clinical diagnosis. The malignancy, metastasis and the nodal status may predict the prognosis of this disease.

INTRODUCTION

Paragangliomas (also known as extra-adrenal pheochromocytomas) are rare neuroendocrine neoplasms which are derived from paraganglia, a diffuse neuroendocrine system dispersed from the skull base to the pelvic floor, and these tumors are observed in patients of all ages. Some of the tumors (named as functional paragangliomas) have been discovered to originate, synthesize, store and secrete catecholamines, which leads to elevated levels of urine/serum catecholamines and the typical clinical symptoms such as episodic headache (72%), sweating (69%), and palpitations (51%). The tumors, which can arise in any area of the body containing embryonic neural crest cells, are mainly composed of chromaffin cells[1,2]. Because of their rarity, little information is available regarding the natural history of these tumors and patient outcome after resection, and especially regarding the diagnosis of malignant tumors. We present a review of 152 cases of resected paragangliomas in our single hospital over a greater than 30-year period and review the relevant literature.

MATERIALS AND METHODS

In this study, data were supplied by West China Hospital, Sichuan University (Department of Pathology, Department of Gastrointestinal Surgery and other relevant departments). From April 1976 through December 2007,

152 patients with paragangliomas (also referred to as extra-adrenal pheochromocytomas) underwent resection at the West China Hospital, Sichuan University. Demographics, survival time, tumor location, surgical treatment, pathological documents and other relevant data were extracted from hospital records and pathological reports. Patient confidentiality was ensured in all cases.

For the purpose of this study, malignant tumors were defined as those associated with identified lymph node metastases, distant metastases, vascular invasion, tumor necrosis and other identified malignant behaviors. The tumor histology was defined and classified as described in the pathological reports. Mortality and survival was calculated based on last follow-up or death. Statistical analyses included survival analysis (Kaplan-Meier method), descriptive statistical analyses and χ^2 analysis. Survival rates were compared using log-rank tests. $P < 0.05$ was considered as statistically significant.

RESULTS

A total of 152 cases of extra-adrenal paraganglioma who underwent resection were identified in West China Hospital, Sichuan University. Amongst these cases were: tumors located in retroperitoneum (85 cases), urinary bladder (6 cases), vertebral canal (7 cases), mediastinum (8 cases), mesostenium (2 cases), lung (3 cases), neck (20 cases, including 8 cases of glomus jugulare tumor, 16 cases of carotid body tumor and 1 case of peripharyngeal tumor), and the rest were tumors located in intracalvarium, liver, supraclavicular fossa, vaginal wall, spermatic cord, sacral bone, greater omentum, nasal cavity, mouth floor, rectum and orbital cavity, respectively. The median age of the 152 patients at the diagnosis was 43 years (range; 8-82 years). Eighty-nine patients were men and 64 patients were women. Of these cases, 30 tumors (19.74%) were diagnosed as malignant paragangliomas, including 6 cases of lymph node metastases, 4 cases of distant metastases (3 cases in femur, 1 case in thoracic vertebra), 1 case of recurrence, 22 cases of local invasion (including vascular invasion), and 2 cases were accompanied by other malignant diseases (rectal cancer and neuroblastoma).

Amongst these 152 cases, paragangliomas from various sites were examined for a host of neural and neuroendocrine markers by immunohistochemistry including neurone specific enolase (NSE), S-100 protein, synaptophysin (Syn), chromogranin A (CgA), Cytokeratin (CK), and vimentin: the results are shown in Table 1. NSE was found to be expressed in nearly all the paragangliomas (benign and malignant), and there was no significant difference between benign or malignant tumors ($P = 0.805$). S100, Syn and CgA were found to be expressed in most of the paragangliomas; again no significant difference was found between benign or malignant tumors. CK was found to be expressed in some of the cases, but no significant difference was identified between benign or malignant tumors as the $P = 0.077$. Vimentin was found to be selectively expressed in benign tumors, and a significant difference between benign and malignant paragangliomas was observed ($P = 0.007$), which may be useful in differentially diagnosing between the malignant and benign tumors. In benign tumors, there was a significant difference in the expression of those markers ($P < 0.001$), and the same result could also be found in the malignant tumors ($P < 0.001$). The difference of the expression of those markers in the tumors could be a clue to the future investigation of the diagnosis.

Table 1 Expression of markers in paragangliomas by immunohistochemistry

Markers	Positive rate in benign groups (%)	Positive rate in malignant groups (%)
NSE	99.0	100.0
S100	84.6	71.4
Syn	85.9	95.0
CgA	92.7	88.2
CK	22.2	5.3
Vimentin	76.9	12.5

The overall 5-year survival in all the 152 cases was 88.82%, while in the malignant group of 30 cases, the five-year survival was 43.33%. The median survival in malignant cases was 50 mo. It could be observed that survival correlated significantly with malignancy ($P < 0.001$, Figure 1A). Furthermore, in the malignant group, survival correlated significantly with the presence of metastasis ($P < 0.001$, Figure 1B), the nodal status ($P < 0.001$, Figure 1C), but not with the local invasion ($P = 0.708$, Figure 1D). The survival analysis revealed a significant correlation between survival and the malignancy, metastasis and the nodal status, which may predict the prognosis of this disease.

DISCUSSION

Histologically, paragangliomas are characterized by a honeycomb pattern in which well-circumscribed nests (Zellballen) of round-oval or giant multinucleated neoplastic cells with cytoplasmic catecholamine granules are surrounded by S-100-positive supratentorial cells. Pleomorphism, mitotic figures, and bizarre nuclear forms (which do not necessarily reflect a higher grade of malignancy) may also be seen. There are no standardized histological criteria for differentiating malignant and benign paragangliomas and pheochromocytomas, and they are considered malignant only when cells with neoplastic characteristics are found in areas in which paraganglionic tissue is normally absent. Also, those tumors that contain large numbers of aneuploid or tetraploid cells, as determined by flow cytometry, are more likely to recur[1,3]. Paragangliomas are of two types, sympathetic and parasympathetic. The tumors usually have their origin in adrenal medulla, the organs of Zuckerkandl, the carotid body, aorticopulmonary, intravagal, *etc*[4,5] (Figure 2).

The paraganglioma cells are discovered to be characterized by the presence of neuroendocrine markers, including neurone specific enolase (NSE), S-100 protein,

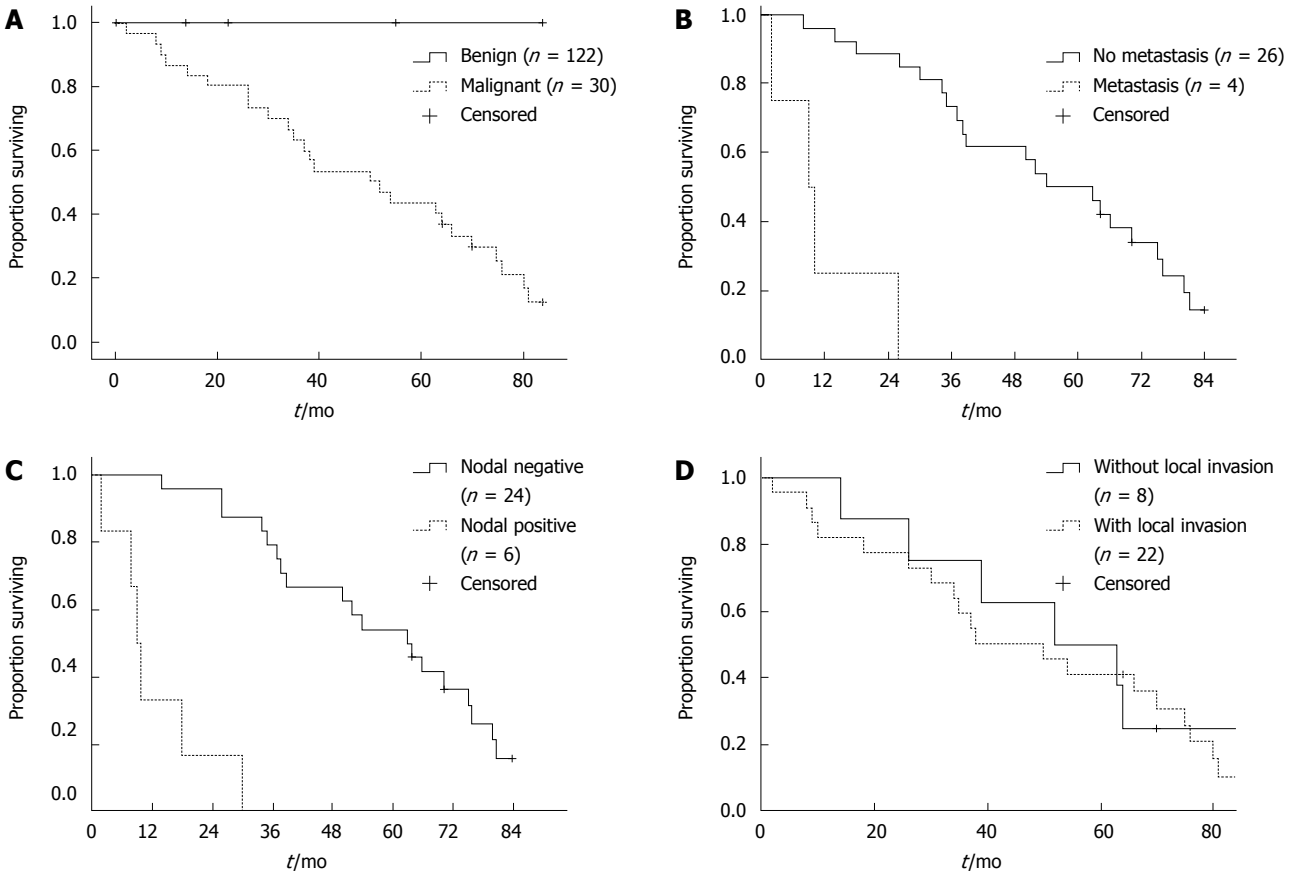

Figure 1 Survival of patients undergoing resection of paraganglioma. Stratified by A: Malignancy ($P < 0.001$); B: Metastasis ($P < 0.001$); C: Nodal status ($P < 0.001$); D: Local invasion ($P = 0.708$).

synaptophysin (Syn), chromogranin A (CgA), Cytokeratin (CK), vimentin, PGP9.5 and CD56, *etc*. The tumor has multiple synthetic activities, and in spite of its heterogeneity, chromogranin A and synaptophysin are the most common neuropeptides synthesised, as they are associated with the presence of neuroendocrine storage granules[6]. The presence of some of these markers in the paragangliomas, as well as their differential expression between the benign and malignant tumors, has been confirmed in our study which may suggest that an immunophenotypic analysis could be useful in the diagnosis of this disease (Figure 3).

Paragangliomas and pheochromocytomas are embryologically related tumors, sharing a neural-crest origin, several clinical features, and an overlapping genetic profile, although they do show variability as to site, histology, and biology. The occurrence of these tumors in familial settings, their association with hereditary syndromes, and their genetic alterations revealed that some cases are familial[7,8]. Multiple endocrine neoplasia type 2 (MEN2), neurofibromatosis type 1 (NF1) and von Hippel Lindau (VHL) syndrome subtypes 2A, 2B, and 2C account for about 10% of cases; the genes involved being the RET proto-oncogene and the NF1 and VHL tumor suppressor genes, respectively. However, with the identification of the familial paraganglioma syndromes, characterized by mutations in the subunits of the succinic dehydrogenase (SDH) enzyme (Table 2, Note: The

Table 2 Classification and characteristics of familial paraganglioma syndromes[8]

		Adrenal	Extra-adrenal Sympathetic	Parasympathetic
Familial paraganglioma 1	SDHD	+	+	+
Familial paraganglioma 3	SDHC			+
Familial paraganglioma 4	SDHB	+	+	+

syndromes were named in the order in which they were identified)[8], it now appears that over 30% of cases are associated with an inherited genetic disposition[8-10]. In our investigation, a very high malignancy rate of 19.74% was obtained, which may suggest that a significant proportion of these patients may have underlying familial SDH gene mutations. This finding also urges us bear in mind the gene mutation test for paraganglioma cases in following investigations. In many previous studies, it has been found that some malignant paraganglioma showed both loss of 8p and gain of 11q13, suggesting these alterations could be markers of malignancy. Furthermore, it has been confirmed that mutations of the SDH and loss of heterozygosity (LOH) on chromosome 11 result in a small fraction of sporadic and almost all familial forms of paraganglioma. The development of paraganglioma in diverse anatomical locations in subjects with SDHB, SDHC, and SDHD germline mutations indicates

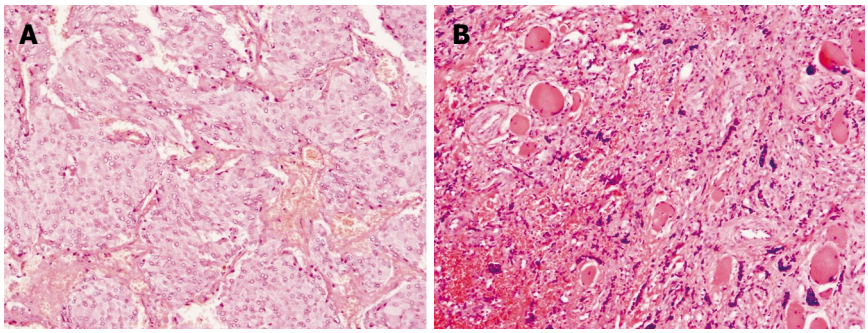

Figure 2 The histologic analysis of paraganglioma. The hematoxylin-eosin stain of chief (type 1) cells arranged in one of the typical cell nests in a retroperitoneal benign tumor (A), and in a femur metastasis (B), (× 50).

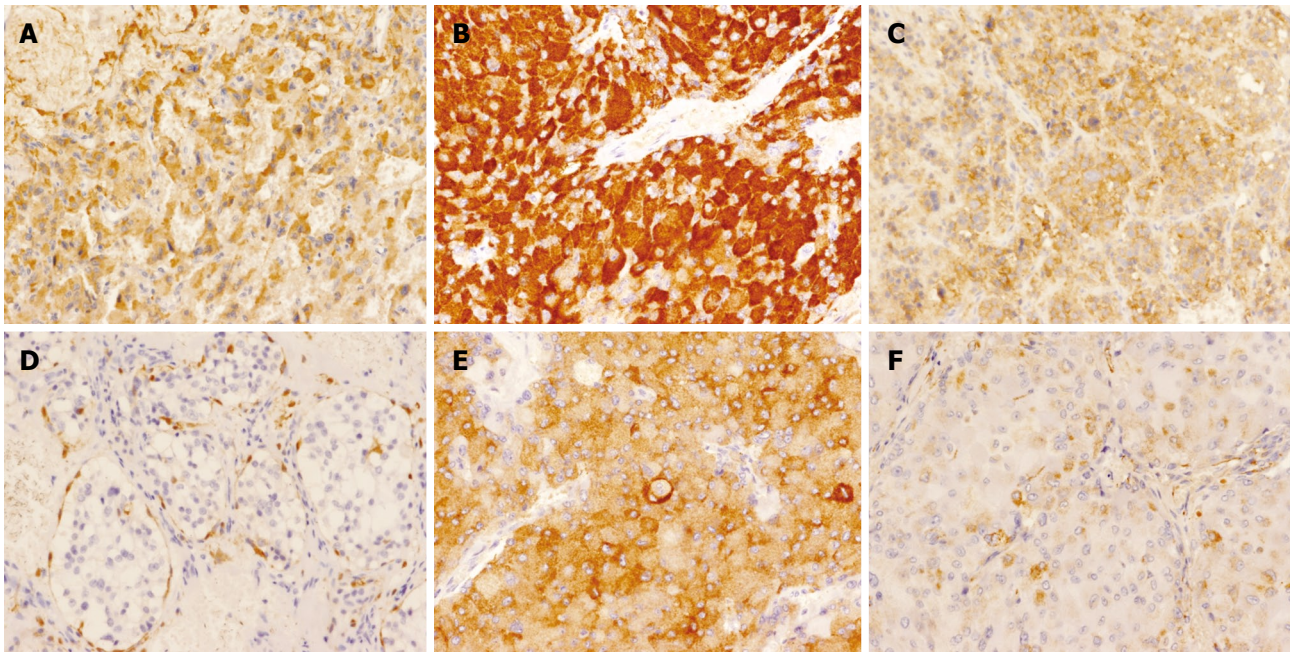

Figure 3 The immunohistochemistry analysis of paraganglioma. The immunolabeling test shows that peripheral to the cell nests are the stellate sustentacular (type 2) cells, intensely brown after S-100 immunolabeling (D), and in some cases CK (A, CgA (B), NSE (C), Syn (E) and vimentin (F) are also positive for paraganglioma, (× 100).

that the paraganglionic system throughout the body is a target for paraganglioma. Thus, the possibility of mitochondrial SDH germline mutations should be raised in the differential diagnosis of all paragangliomas. Whether certain subunit mutations are more strongly associated with a given anatomical location, hormonal activity, malignancy, age at onset, tumor multiplicity, and tumor size remains to be established. Hereditary paraganglioma is closely tied in with germline mutations affecting genes encoding for the SDH enzyme system, which is a heterotetrameric complex with functions in the Krebs cycle related to the oxidation of succinate to fumarate leading to ATP production. The catalytic subunits of SDH are encoded by the genes SDHD, SDHB, SDHC, and SDHA. SDHD encodes the smallest subunit of SDH. SDHA and SDHB are anchored to the mitochondrial inner membrane through membrane-spanning subunits encoded by SDHC and SDHD. A large number of mutations (approx 30) in hereditary paraganglioma have been described for SDHD and SDHB each[3,11,12]. Germline mutations in SDH genes are associated with the development of paraganglioma in diverse anatomical locations, a finding that has important implications for the clinical management of patients and genetic counseling of families. Consequently, patients with paraganglioma and a SDH germline mutation should be diagnosed as potentially hereditary, regardless of family history, anatomical location, or multiplicity of tumors.

In the past, studies involving biochemical measurements of urine/plasma catecholamines were found to have appropriate sensitivity and specificity for detecting catecholamine-secreting functional paragangliomas. Among all the tests studied, measurements of plasma free metanephrines were found to have the best predictive value for excluding or confirming a pheochromocytoma or functional paraganglioma[13,14]. As for those nonfunctional paragangliomas, because they are a heterogeneous group of tumors, using a single test may not be reliable, and a combination of tests may result in higher diagnostic accuracy. Furthermore, scintigraphy with iodide 131-labeled metaiodobenzylguanidine (MIBG 131) is found to be useful, not only for noninvasive diagnosis of paragangliomas, but also for palliative treatment in cases for which other types of treatment have been unsuccessful[15]. Positron emission tomography (PET)-scanning with [18F]-fluorodeoxyglucose, [11C]-

Table 3 Pheochromocytoma of the adrenal gland scoring scale (PASS)[22]

Feature	Score if present (No. of points assigned)
Large nests or diffuse growth (> 10% of tumor volume)	2
Central (middle of large nests) or confluent tumor necrosis (not degenerative change)	2
High cellularity	2
Cellular monotony	2
Tumor cell spindling (even if focal)	2
Mitotic figures > 3/10 HPF	2
Atypical mitotic figure(s)	2
Extension into adipose tissue	2
Vascular invasion	1
Capsular invasion	1
Profound nuclear pleomorphism	1
Nuclear hyperchromasia	1
Total	20

HPF: High-power field.

hydroxyephedrine, or 6-[18F] fluorodopamine may help to identify conventionally undetectable tumors[16-19]. For the diagnosis of malignancy in paraganglioma, the clinical and histological features predictive of malignancy are still poorly defined for this type of tumor; the level of serum catecholamines does not necessarily correlate with the malignancy of the tumor. In most previous literature, immunohistochemistry has not been of use for refining the diagnosis of malignant potential, though some malignant tumors may express fewer or different peptides than benign. S100-positive sustentacular cells are often sparse in malignant tumors, although this test is not 100% sensitive[3,6,20]. The only absolute criterion for malignancy is the presence of metastases to sites where chromaffin tissue is not usually found. However, some other features of the tumor may indicate the malignant characteristic, including recurrence, gross local invasion, vascular or capsular invasion, tumor necrosis, malignant histological pattern and cellularity[21]. Also, some evidence suggests that multifactorial scoring systems can help to histopathologically discriminate tumors which pose a significant risk of metastasis from those that do not. In 2002, Thompson[22] proposed the PASS system (Pheochromocytoma of Adrenal Scaled Score), which scores multiple microscopic findings (Table 3). A PASS of < 4 accurately identified all histologically benign and clinically benign tumors. A PASS of $\geqslant 4$ correctly identified all tumors that were histologically malignant. In our study, vimentin was found to be selectively expressed in benign tumors, which may be useful in the differential diagnosis between the malignant and benign tumors. However, in the PASS system as Thompson[22] proposed, vimentin has not been involved and scored as a neuroendocrine marker in the diagnosis of this disease. In order to confirm the value of vimentin, following investigations should involve more cases and areas, more precise testing techniques such as the quantitation test of RNA and protein should be involved, as well. If the diagnostic value of vimentin could be confirmed in the future, vimentin should indisputably be added into the PASS system.

Due to the rarity of this disease, the outcomes of survival analysis in different research studies are not highly coherent. In benign cases, most studies showed that the 5-year survival rate is above 95%; recurrences occur in less than 10% of cases. Whereas, due to the lower incidence of malignant paragangliomas, there are single case reports rather than larger scale case studies and clinical controlled randomized tests, and the exact survival in the malignant cases is difficult to approach. Similar to our study, the value has been found to be 43.33%. The survival in malignant cases was thought to be related to the familial circumstance, the stage of the disease at the diagnosis, the therapeutic methods and follow-up after the surgery, which has also been identified by our study[23,24]. In our study, it was also confirmed that the survival in malignant cases was obviously lower than the benign group as previously reported in many articles. Furthermore, it was revealed that the non-chromaffin site metastases and nodal metastases distinct from local invasion played an important role in the progression and prognosis of this disease, which may help in the estimation of survival in each case and moreover offer individualized therapy for each patient. This result also means that following studies should investigate the possible unique mechanism of metastasis and nodal invasion of this disease, so that the method to prevent this progress may be found.

COMMENTS

Background

Paraganglioma is a rare neuroendocrine neoplasm. This disease usually originates from a diffuse neuroendocrine system dispersed from the skull base to the pelvic floor. Paraganglioma is observed in patients of all ages. Some of the tumors (named as functional paragangliomas) have been discovered to originate, synthesize, store and secrete catecholamines, which leads to elevated levels of urine/serum catecholamines and the typical clinical symptoms such as episodic headache (72%), sweating (69%), and palpitations (51%). Due to the rarity of this disease, the natural history of these tumors, patient outcome after resection, and especially the diagnosis of malignant tumors are still under investigation.

Research frontiers

Nowadays, there are no standardized histological criteria for differentiating malignant and benign paragangliomas, and they are considered malignant only when cells with neoplastic characteristics are found in areas in which paraganglionic tissue is normally absent. The paraganglioma cells are discovered to be characterized by the presence of neuroendocrine markers, including neurone specific enolase (NSE), S-100 protein, synaptophysin (Syn), chromogranin A (CgA), Cytokeratin (CK), vimentin, PGP9.5 and CD56, *etc*. The differential expression of those markers between the benign and malignant tumors and the value of them in the differential diagnosis are broadly discussed.

Innovations and breakthroughs

A Pheochromocytoma of Adrenal Scaled Score (PASS) system was proposed for making the differential diagnosis between malignant cases and benign cases. This system scores multiple microscopic findings such as tumor necrosis, high cellularity, cellular monotony, vascular invasion, *etc*. The differential expression of some markers between the benign and malignant tumors has been confirmed in many studies, which may suggest that immunophenotypic analysis could be useful in the diagnosis of this disease.

Applications

In the study, vimentin was found to be selectively expressed in benign tumors, which may be useful in the differential diagnosis between the malignant and benign tumors. The high malignancy rate of 19.74% may suggest that a significant proportion of these patients have underlying familial SDH gene

mutations. This finding may also attract more attention to the gene mutation test for paraganglioma cases in following investigations. The survival data may help clinicians to estimate the survival in each case and moreover offer individualized therapy for each patient.

Terminology

Paraganglioma (also known as extra-adrenal pheochromocytoma) is a rare neuroendocrine neoplasm which is derived from paraganglia, a diffuse neuroendocrine system dispersed from the skull base to the pelvic floor. Vimentin is a member of the intermediate filament family of proteins. Intermediate filaments are an important structural feature of eukaryotic cells. They, along with microtubules and actin microfilaments, make up the cytoskeleton. Although most intermediate filaments are stable structures, in fibroblasts, vimentin exists as a dynamic structure.

Peer review

This manuscript provides relevant data on the rare condition of paraganglioma, especially in the west region of China, which has significant usefulness for clinicians and pathologists alike. The finding about the differential expression of vimentin may be important in the diagnosis of this disease. The survival analysis may help the clinicians to make clinical decisions.

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Influence of heme oxygenase-1 expression on immune liver fibrosis induced by cobalt protoporphyrin in rats

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Abstract

AIM: To investigate the effect of heme oxygenase-1 (HO-1) expression on immune liver fibrosis induced by cobalt protoporphyrin (CoPP) in rats.

METHODS: An immune liver fibrosis model of rat was established by administering human serum albumin (HSA). The rats were divided into CoPP, liver fibrosis and normal control groups. Rats in the CoPP group received intraperitoneal CoPP concurrently with HSA. Expression of HO-1 protein was observed by Western blotting and immunohistochemistry. Hematoxylin and eosin (HE) staining was performed to assess fibrosis proliferation and distribution, proliferation extent of fibroblasts, and alterations in hepatocytes and inflammatory cells. Type Ⅰ and Ⅲ collagens were detected with Van Gieson's (VG) staining and Foot's reticular fiber staining, respectively. In addition, spindle-shaped cells existing at perisinusoidal locations beyond portal and septa areas were investigated with HE staining.

RESULTS: Western blotting and immunohistochemistry showed that the expression of HO-1 protein was higher in the CoPP group than in the liver fibrosis group ($P < 0.05$). Compared with the liver fibrosis group, the serological index of hepatic fibrosis in the CoPP group decreased significantly ($P < 0.05$). HE, VG and Foot's staining revealed that administration of CoPP reduced the extent of hepatic fibrosis. The levels of serological indicators and the number of spindle-shaped cells at perisinuous locations beyond the portal and septa areas were reduced in the CoPP group. Only a few inflammatory cells were seen around the portal areas and central veins in the CoPP group.

CONCLUSION: Increased endogenous HO-1 may suppress liver fibrosis by protecting liver cells, inhibiting inflammatory cell infiltration and hepatic stellate cell transformation.

Key words: Heme oxygenase-1; Cobalt protoporphyrin; Immune liver fibrosis; Rats

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INTRODUCTION

Heme oxygenase-1 (HO-1) and heme degradation products of biliverdin, bilirubin, CO and free iron play an important role in many physiological and pathological processes, such as liver ischemia-reperfusion injury, liver transplantation, and acute liver injury[1-5]. In the present study, we investigated the effects of HO-1 expression on immune liver fibrosis induced by cobalt protoporphyrin (CoPP) in rats.

MATERIALS AND METHODS

Animals

Healthy male Sprague-Dawley rats, weighing 220-270 g,

were obtained from the Laboratory Animal Center of Dalian Medical University.

Reagents

CoPP injections were prepared by dissolving the compound in 0.2 mmol/L NaOH, adjusting its pH value to 7.4 with 1 mmol/L HCl, and diluting it with 0.9% NaCl to a final concentration of 1 mg/mL, as previously described[6]. Twenty percent human serum albumin (HSA) injection (Instituto Grifols, S.A. Barcelona, Spain), Freunds' incomplete adjuvant (Santa Cruz Biotechnology, Santa Cruz, CA, USA), protoporphyrin IX cobalt chloride (Sigma, St Louis, MO, USA), rabbit anti-HO-1 (Boster Biological Technology, Wuhan, China), and anti-mouse IgG, anti-rabbit IgG (Zhong Shan Golden Bridge Biotechnology, Beijing, China) were used in the study.

Animal model and grouping

Animals were divided randomly into a liver fibrosis group (n = 20), a CoPP group (n = 20), and a normal control group (n = 12). An immune liver fibrosis rat model was established as previously described[7]. Rats were allergized with HSA, and then injured by injecting albumin into the tail vein (2.5 mg per rat each time and increased gradually to 4.5 mg, twice weekly for 6 wk). Rats in the CoPP group received intraperitoneal CoPP (5 mg/kg) concurrently with HSA administration.

Sample collection

Rats were attacked by HSA for 6 wk, fasted for 12 h, and then weighed. Three percent sodium pentobarbital injection (2 mL/kg) was used as an anesthetic agent. Four milliliters of blood was collected from the eyeball of rats and stored at -20℃. Livers were removed from the rats, fixed in a 10% neutral formalin solution, embedded in paraffin, and preserved at -80℃.

Western blotting

One milliliter of lysate containing 20 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 1%Triton X-100, 1 mmol/L phenylmethanesulfonylfluoride, was added to 100 mg liver tissue. The mixture was homogenized by centrifugation at 12000 r/min for 3-5 min at 4℃, and the supernatant was separated. After SDS-PAGE, the sample was transferred to a polyvinylidine fluoride membrane and stained with 3,3' diaminobenzidine. The sample was incubated with primary antibody (rabbit-anti-mouse HO-1 monoclonal antibody, 1:100) and secondary antibody (peroxidase-labeled sheep-anti-rabbit antibody, 1:100), with β-actin as an internal reference.

Immunohistochemical analysis

Paraffin-embedded liver tissue was cut into sections, which were routinely stained with HE. Cells in good condition were stained using an immunohistochemical method (streptavidin-peroxidase method), after dewaxing, hydration, inactivation, incubation with primary antibody (rabbit-anti-mouse HO-1 monoclonal antibody; 1:150) and secondary antibody (biotin-labeled sheep-anti-rabbit antibody; 1:100), and mounting. The primary antibody was administered with PBS to serve as a negative control. Yellow material in the cytolymph was considered to be a positive cell. Five high-power microscopic fields were randomly chosen per slice. The percentage of positive cells increased as the intensity of staining increased in every field. Ultimately, the average of five fields was used to compare differences between groups.

Measurement of biochemical indicators

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were measured with an automatic biochemical analyzer.

Measurement of liver fibrosis indicators

The levels of serum hyaluronic acid (HA), laminin (LN), type Ⅲ procollagen (PC Ⅲ) and type Ⅳ collagen (Ⅳ-C) were measured using a gamma radioimmunoassay counter.

Pathological analysis

Proliferation and distribution of liver fibrous tissue, proliferation of fibroblasts and changes in liver cells, portal areas and central veins were observed by HE staining[8]. Spindle-shaped cells existing at the perisinuous location beyond the portal and septal areas were counted. The proliferation degree of type Ⅰ and Ⅲ collagen was observed with Van Gieson's (VG) and Foot's staining, respectively[8].

Statistical analysis

Data analysis was performed using SPSS 10.0 software (Chicago, IL, USA). Analysis of variance (ANOVA) or Wilcoxon statistical methods were used to determine statistical significance. The results were expressed as mean ± SD. $P < 0.05$ was considered statistically significant.

RESULTS

Western blotting for HO-1 expression

The HO-1 protein expression level was significantly higher in the liver fibrosis group than in the normal control group ($P < 0.01$) and higher in the CoPP group than in the liver fibrosis group ($P < 0.05$, Figure 1).

Immunohistochemistry

Liver cells were not or only slightly stained in the normal control group (Figure 2A). Liver cells and mesenchymal cells were diffusely and unevenly stained in the liver fibrosis group (Figure 2B) and diffusely but strongly stained in the CoPP group (Figure 2C). Compared with the liver fibrosis group, the staining intensity and scope increased significantly in the CoPP group. The score for HO-1 protein expression was 0.80 ± 0.79 in the normal control group, 4.00 ± 1.31 in the liver fibrosis group, and 5.52 ± 1.15 in the CoPP group. The score for HO-1 protein expression was significantly higher in the CoPP group than in the liver fibrosis group ($P < 0.05$).

CoPP reduced the degree of liver fibrosis

The level of serological indicators was significantly

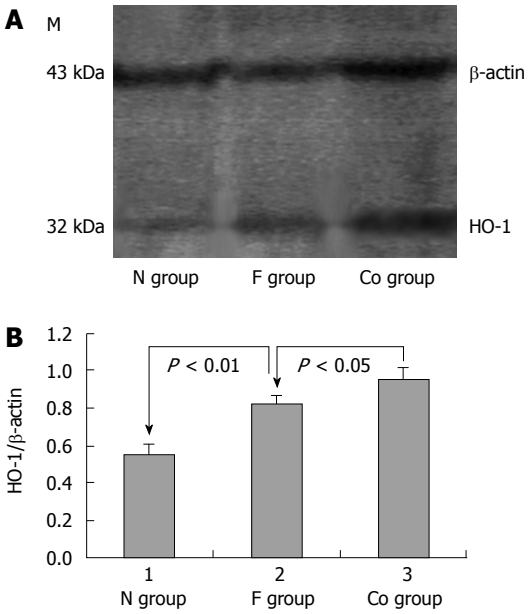

Figure 1 Western blotting of HO-1 protein expression (A) and densitometric analysis of HO-1/β-actin expression (B) in rat liver tissue.

higher in the liver fibrosis group than in the normal control group ($P < 0.01$), and significantly lower in the liver fibrosis group than in the CoPP group ($P < 0.05$, Table 1). The structure of hepatic lobules seemed integral without fibrous hyperplasia, and some collagen fibers were observed in the portal areas of the normal control group (Figure 3). Significant fibrous hyperplasia and fibrosis extension in the portal areas with fibroblast proliferation, widened lobular septa with more fiber deposition, and formation of fibrous septa were found in the liver fibrosis group (Figure 3). Compared with the liver fibrosis group, fibrous hyperplasia was significantly reduced and fine fibers were seen occasionally and distributed mainly in the portal areas of the CoPP group. The number of fibroblasts was also decreased in the CoPP group (Figure 3). Compared with the normal control group, there was a significant increase in fibrosis and hyperplasia of fibroblasts, and type Ⅰ and Ⅲ collagens in the liver fibrosis group ($P < 0.01$). The extent of fibrosis was lower in the CoPP group than in the liver fibrosis group (Table 2).

Mechanism underlying the effect of HO-1 on immune liver fibrosis

Injection of HSA increased serum ALT and AST levels ($P < 0.01$). The serum ALT and AST levels were lower in the CoPP group than in the liver fibrosis group ($P < 0.05$, Table 3). Rat liver cells were relatively uniform, yet most were swollen with a small amount of fatty degeneration and few nuclei were strongly stained and dissolved in the liver fibrosis group. In the CoPP group, most liver cells were normal. Only a small number were swollen and no degeneration of cells was observed, suggesting that HO-1 expression can protect impaired hepatocytes at a certain extent. The number of spindle-shaped cells was 37.2 ± 4.7 in the normal control group, 55.2 ± 3.5 in the liver fibrosis group, and 50.6 ± 3.6 in the CoPP group ($P < 0.01$). Meanwhile, the number of spindle-shaped cells, which were confirmed by electron microscopy to be activated hepatic stellate cells (HSCs), was reduced in the CoPP group. In the liver fibrosis group, a large number of inflammatory cells infiltrated the portal areas. Most of them were lymphocytes, and a small number were neutrophils. A small number of inflammatory cells were observed in the CoPP group.

Table 1 Levels of serum HA, LN, type Ⅲ precollagen (PC Ⅲ) and type Ⅳ collagen (Ⅳ- C) in different groups

Group	*n*	PCⅢ (ng/mL)	Ⅳ-C (ng/mL)	LN (ng/mL)	HA (ng/mL)
N	11	31.77 ± 9.47	28.65 ± 5.29	28.74 ± 9.73	67.27 ± 15.21
F	15	51.11 ± 11.86[b]	48.29 ± 8.93[b]	47.56 ± 17.65[b]	92.62 ± 18.61[b]
Co	17	43.10 ± 8.91[a]	41.04 ± 8.19[a]	38.54 ± 7.39[a]	79.88 ± 13.65[a]

N group: Normal control group; F group: Liver fibrosis group; Co group: CoPP group. [a]$P < 0.05$ *vs* liver fibrosis group; [b]$P < 0.01$ *vs* normal control group.

Table 2 Pathological grading of rat fibrous tissue

Group	*n*	-	+	++	+++
HE staining					
N	11	11	0	0	0
F	15	0	8	7	0
Co	17	0	15	2	0
Foot staining					
N	11	11	0	0	0
F	15	0	4	8	3
Co	17	0	10	7	0
VG staining					
N	11	11	0	0	0
F	15	0	7	6	2
Co	17	0	14	3	0

Table 3 Levels of serum ALT and AST in different groups

Group	No.	ALT (U/L)	AST (U/L)
N	11	52.55 ± 9.36	176.45 ± 11.48
F	15	70.40 ± 12.30[b]	210.27 ± 11.50[b]
Co	17	60.82 ± 8.59[a]	194.53 ± 11.98[a]

[a]$P < 0.05$ *vs* CoPP group, [b]$P < 0.01$ *vs* normal control group.

DISCUSSION

Many chronic liver diseases progress to liver fibrosis[9]. The core aspect of the occurrence and development of hepatic fibrosis is to activate HSCs and transform them into myofibroblast-like cells[10,11]. Various factors result in hepatic fibrosis. Interactions between cells, cells and matrix, or transmitters and matrix, constitute a complex network that underlies the occurrence and development of liver fibrosis[12]. If the cause could be suppressed and the network systems could be impeded, hepatic fibrosis or cirrhosis could be effectively prevented.

Heme is catalyzed into CO, biliverdin and free iron

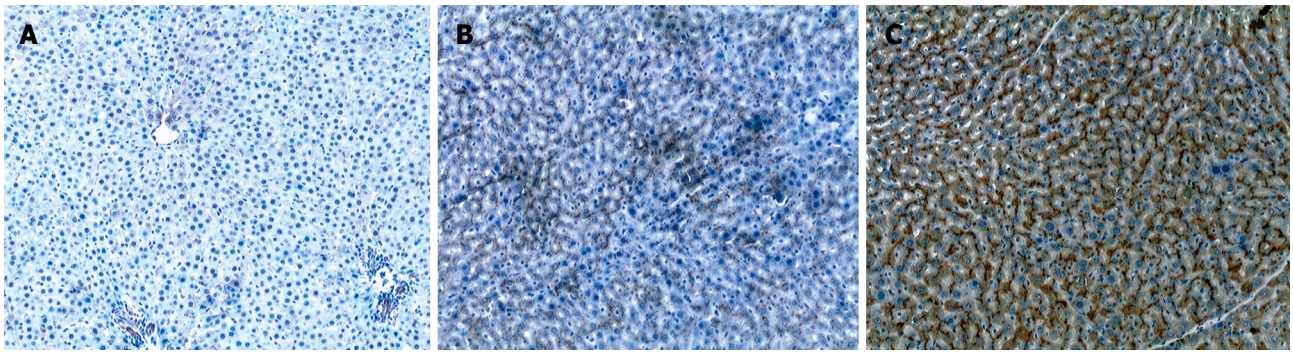

Figure 2 HO-1 protein expressions in rat liver tissue of normal control group (A), liver fibrosis group (B), and CoPP group (C) (× 100).

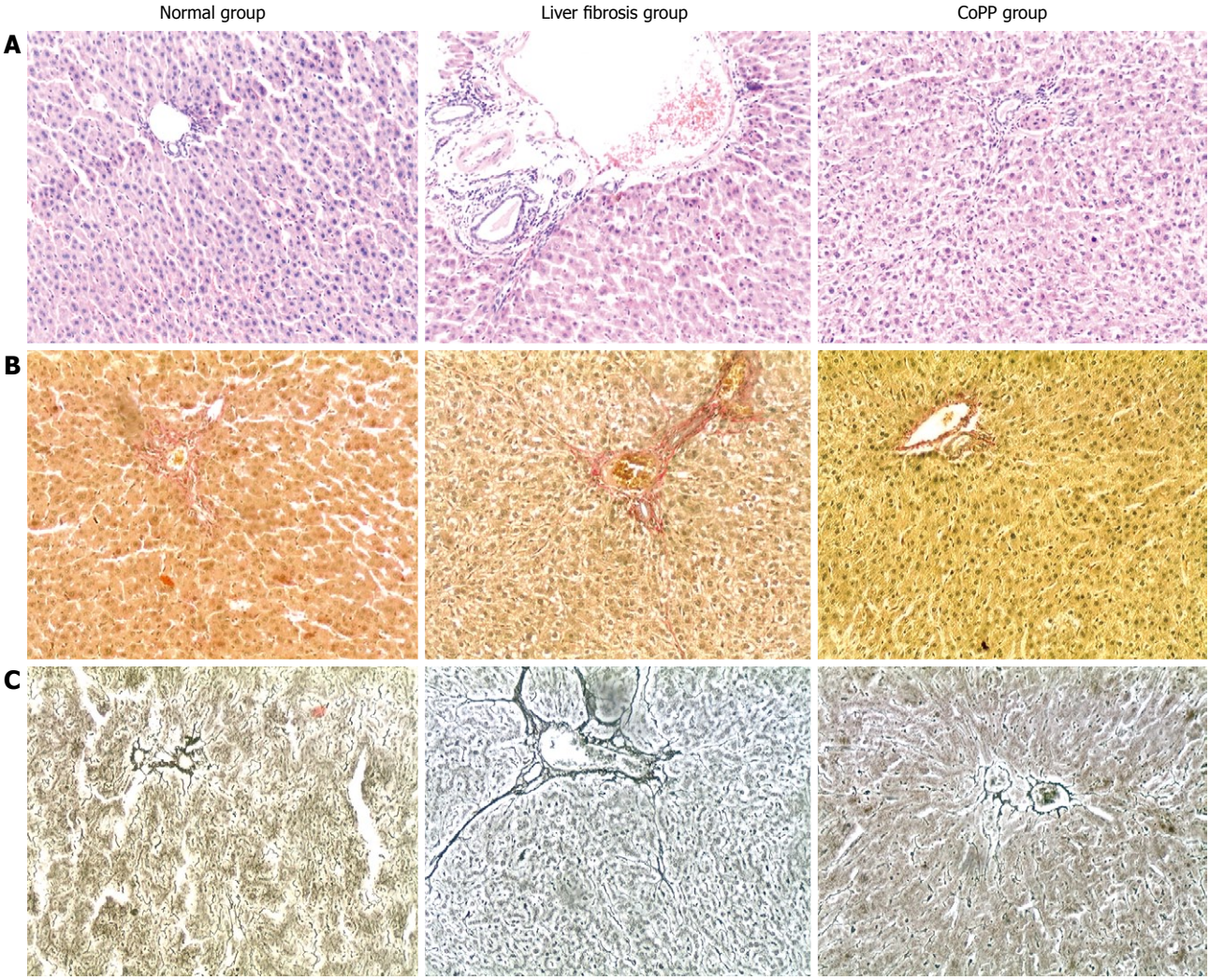

Figure 3 HE staining (A), VG staining (B), and Foot's staining (C) for liver tissues from rats in different groups (× 100).

by HO-1, a rate-limiting enzyme in heme metabolism. HO-1 expression can be induced by various stress factors, such as oxidative stress, inflammatory factors and heavy metals. In addition, some protoporphyrins, especially CoPP, can increase HO-1 expression *in vivo*. HO-1, as a protective protein *in vivo*, plays a vital role in many aspects, such as anti-oxidative stress, anti-inflammation, anti-cell proliferation, and regulation of cytokine expression.

HO-1 is only expressed in Kupffer cells (KCs) of normal liver tissue. In hepatic cirrhosis, however, it is expressed mainly in KCs and fibroblasts[13,14]. HO-1 can inhibit HSC proliferation and type I collagen mRNA expression in cultured human liver fibroblasts. When the adeno-associated-virus-mediated HO-1 gene is injected into portal veins of rats with micronodular cirrhosis induced by CCl_4, HO-1-transduced HSCs can reduce type I collagen transcription, proliferation ability and macrophage infiltration, thus improving the biochemical function of the liver[15,16].

The main cause for chronic liver disease in China

is hepatitis B virus (HBV) infections HBV cannot be used to establish animal models of liver fibrosis. Some scholars believe that rat models of immune liver fibrosis induced by HSA have some similarities in morphology and pathogenesis to liver fibrosis caused by HBV[17]. Therefore, it is of great significance to study the effects of HO-1 on immune liver fibrosis.

In the present study, HO-1 protein expression was observed in the CoPP group. Type Ⅲ collagen was significantly increased early in hepatic fibrosis. Type Ⅰ collagen fibers are mainly distributed at the fibrous septa, while type Ⅲ collagen fibers are associated with the reticular fibers[18,19]. In addition, serum HA, PC Ⅲ, LN and Ⅳ-C levels are closely correlated with liver fibrosis[20,21], indicating that increased HO-1 expression can suppress the occurrence and development of liver fibrosis.

We investigated preliminarily the effect of HO-1 expression on immune liver fibrosis induced by CoPP in rats. When liver cells were destroyed, the membrane permeability was increased and the mitochondria were damaged, and the activity of AST and ALT was elevated. The extent of damage to liver cells is consistent with the level of enzymatic activity[22]. Compared with the liver fibrosis group, serum AST and ALT levels were significantly increased in the CoPP group, indicating that HO-1 protects liver cells. Wen *et al*[23] have established a liver injury model induced by D-galactosamine and lipopolysaccharide (LPS), and found that pretreatment with hemoglobin increases HO-1, reduces damage to the liver, and lowers serum ALT and AST levels, thus improving liver disease. Nakahira *et al*[24] have shown that HO-1 activity inhibited by tin-protoporphyrin can lead to severe liver damage and significantly increased ALT levels. Moreover, CO also exerts protective effects on liver cells. Amersi *et al*[25] have shown that CO can protect liver cells, the AST levels were significantly lower, and the liver structure was normal without swelling or necrosis in the experimental group. Both biliverdin and bilirubin are antioxidants. When biliverdin is transformed into bilirubin, it can strengthen the oxidative capacity, while bilirubin protects the lipid bimolecular layer of the cell membrane[26], suggesting that HO-1 protects liver cells by consuming oxygen molecules and reducing oxygen free radicals, and prevents the peroxidation of free radicals in membrane lipids and maintains membrane integrity and normal physiological functions.

A high HO-1 expression level induced by CoPP plays an important role in reduction of the inflammatory response of liver cells. During inflammatory responses, local blood flow is reduced and leukocytes adhere to activated endothelial cells, thus releasing enzymes and causing cell injury. The key step is adhesion between leukocytes and endothelial cells. Selectin and cell adhesion factors are also involved in the process.

Anti-inflammatory effects of HO/CO are achieved by reducing the expression of adhesion molecules[27]. Macrophages are also activated in inflammatory responses, and produce arachidonic acid substances, tumor necrosis factor-α (TNF-α), and other inflammatory mediators that act on the liver and cause liver injury. LPS can stimulate KCs to generate inflammatory mediators. CO may prevent the expression of inflammatory cytokines [i.e. TNF-α and interleukin (IL)-1β][28], inhibit the secretion of IL-2[29], and reduce T-cell proliferation, thus achieving an anti-inflammatory effect. In addition, CO can increase the expression of anti-inflammatory cytokine IL-10, which can induce HO-1 expression, allowing HO-1 to produce more CO[30]. Thus, a reduction of inflammation induced by HO-1 is achieved by decreasing the activation of KCs, which suppresses the migration and adhesion of leukocytes, and inhibits the release of inflammatory mediators, finally relieves the inflammatory damage to endothelial cells.

A high HO-1 expression level induced by CoPP can inhibit the transformation of HSCs to fibroblasts. In the present study, the number of spindle-shaped cells was decreased in the CoPP group and increased in the liver fibrosis group. Most of these cells were activated HSCs, indicating that HO-1 can inhibit HSC activation. The fibroblasts of α-smooth muscle that express actin are transformed from dormant HSCs to live epithelial or matrix cells. Irrespective of its origin, the activity of fibroblasts is associated with cytokines and chemotactic factors secreted by macrophages[31]. Normal liver cell membranes have a contact-inhibition effect on the proliferation of HSCs and KCs. When liver cells are damaged, destruction of the cell membrane leads to loss of the contact-inhibition effect on HSCs, thus leading to activation of HSCs. The more HSCs are activated, the more cytokines are secreted (including insulin-like growth factor-1). Finally, the feed-forward cycle prevents the reversal of liver fibrosis. In short, HO-1 may inhibit the activation of HSCs.

In conclusion, HO-1 inhibits liver fibrosis and is closely related to other liver diseases. Further study is needed to elucidate its protective effect on immune liver fibrosis.

COMMENTS

Background

Heme oxygenase-1 (HO-1) and heme degradation products are involved in acute liver injury. However, their influence on chronic liver injury is still unclear.

Research frontiers

There are still many unclear or confusing problems about the development and progression of liver cirrhosis. These problems result in difficulties in the treatment of cirrhosis. It is of great significance to study the effects of HO-1 on immune liver fibrosis. The expression of HO-1 induced by drugs may provide a promising way to treat successfully liver fibrosis.

Innovation and breakthroughs

By establishing a model of immune liver fibrosis in rats, the authors investigated the effect of HO-1 induced by cobalt protoporphyrin (CoPP), which may provide a new treatment modality for liver fibrosis.

Applications

The expression of HO-1 induced by drugs in target organs may provide a promising way to treat liver fibrosis.

Terminology

HO-1 stands for heme oxygenase-1, which is a rate-limiting enzyme. It is also known as heat shock protein 32, and is susceptible to various stress factors such as oxidative stress, inflammatory factors, and heavy metals. In addition, some protoporphyrins, especially CoPP can increase HO-1 expression *in vivo*.

Peer review

The study is interesting. The authors showed that increased expression of

HO-1 could suppress liver fibrosis, thus providing a new treatment modality for liver fibrosis.

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Survival predictors in patients treated with a molecular adsorbent recirculating system

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Abstract

AIM: To identify prognostic factors for survival in patients with liver failure treated with a molecular adsorbent recirculating system (MARS).

METHODS: MARS is a liver-assisting device that has been used in the treatment of liver failure to enable native liver recovery, and as a bridge to liver transplantation (LTX). We analyzed the 1-year outcomes of 188 patients treated with MARS, from 2001 to 2007, in an intensive care unit specializing in liver disease. Demographic, clinical and laboratory parameters were recorded before and after each treatment. One-year survival and the number of LTXs were recorded. Logistic regression analysis was performed to determine factors predicting survival.

RESULTS: The study included 113 patients with acute liver failure (ALF), 62 with acute-on-chronic liver failure (AOCLF), 11 with graft failure (GF), and six with miscellaneous liver failure. LTX was performed for 29% of patients with ALF, 18% with AOCLF and 55% with GF. The overall 1-year survival rate was 74% for ALF, 27% for AOCLF, and 73% for GF. The poorest survival rate, 6%, was noted in non-transplanted patients with alcohol-related AOCLF and cirrhosis, whereas, patients with enlarged and steatotic liver had 55% survival. The etiology of liver failure was the most important predictor of survival ($P < 0.0001$). Other prognostic factors were encephalopathy ($P = 0.001$) in paracetamol-related ALF, coagulation factors ($P = 0.049$) and encephalopathy ($P = 0.064$) in non-paracetamol-related toxic ALF, and alanine aminotransferase ($P = 0.013$) and factor V levels ($P = 0.022$) in ALF of unknown etiology.

CONCLUSION: The etiology of liver disease was the most important prognostic factor. MARS treatment appears to be ineffective in AOCLF with end-stage cirrhosis without an LTX option.

Key words: Molecular adsorbent recirculating system; Prognostic factors; Acute liver failure; Acute-on-chronic liver failure; Liver transplantation

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INTRODUCTION

Since its introduction in 1993, molecular adsorbent recirculating system (MARS) albumin dialysis[1,2] has been a subject of research, with the hope of using it to treat effectively patients with rapidly failing liver function. Even though MARS treatment cannot fully compensate for the synthetic and metabolic functions of a normal liver, it has been used as a bridging treatment to sustain the patient until a suitable graft becomes available, or the native liver recovers. MARS treatment has also been used for patients who have a contraindication to transplantation or when a suitable organ is not available.

While the effect of MARS treatment on patient outcome, and laboratory and clinical parameters has been investigated widely in various uncontrolled case series, only a handful of randomized studies have been published[5-9]. Thus, we are still searching to identify which patients are most likely to benefit from this treatment, and to determine whether MARS treatment does in fact improve survival in patients with liver failure. It is crucial to identify not only those patients who have a good possibility of benefiting from MARS treatment, but also those for whom MARS treatment is a futile tool that serves only to prolong suffering when death is imminent.

The aim of this prospective observational study was to identify prognostic factors associated with survival in MARS-treated patients with life-threatening liver failure.

MATERIALS AND METHODS

This was an uncontrolled, prospective, single-center, observational study of 188 consecutive patients who underwent MARS treatment in a liver-disease-specialized intensive care unit (ICU) from May 2001 to March 2007. Four patients were treated before LTX, and then later on after LTX because of graft failure. All patients were categorized into four main groups according to the etiology of liver failure: acute liver failure and injury (ALF), acute-on-chronic liver failure (AOCLF), liver graft failure (GF), or liver failure of miscellaneous etiology. For the final analysis of results, these groups were further divided into subgroups according to specific etiology (Figure 1). Our tertiary liver-disease-specialized ICU is the only transplantation center in Finland.

Patients included in the ALF group required ICU admission and had rapid development of hepatic synthetic dysfunction[10], with or without encephalopathy, and no previous history of liver disease. AOCLF was defined as previously well-compensated chronic liver disease in which an acute decompensation of liver function developed rapidly, as a result of various secondary causes[11]. Graft failure included early (primary dysfunction or non-functioning graft) and late (primarily chronic rejection) dysfunction. The miscellaneous etiologies group contained patients with acute hemorrhagic pancreatitis with ALF, ischemic injury to the liver following myocardial infarction, multiple trauma including injury to the liver, and post-liver resection hepatic failure.

Monitoring and standard medical therapy

All patients received the same standard medical therapy. Blood pressure was monitored *via* arterial and central venous catheters; a Swan-Gantz catheter was used if necessary. All potentially nephro- and hepatotoxic medications were discontinued. Mean arterial pressure was maintained above 65 mmHg with fluid resuscitation and vasoactive medication (primarily noradrenaline infusion). Surveillance for infection and prophylactic antibacterial and antifungal therapy was administered. The level of consciousness was monitored closely and sedatives were avoided in non-intubated patients. If the grade of hepatic encephalopathy was $\geqslant$ 3 according to the West Haven

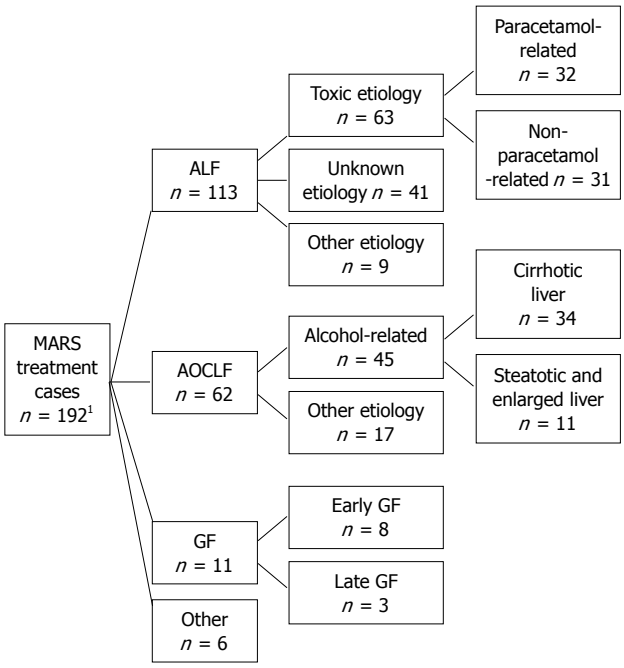

Figure 1 Distribution of liver failure etiologies in MARS-treated patients. [1]192 MARS treatment cases and 188 patients were included in this study. Four patients were treated at two different time points, first due to the primary liver failure, and second for graft failure following LTX.

criteria[12], the patient was usually sedated, intubated, and mechanically ventilated. A standard regimen of lactulose and proton pump inhibitors was used, and the target blood glucose level was normoglycemia, which was maintained with glucose and insulin infusion. *N*-acetylcysteine was used when necessary. Enteral nutrition was employed if possible. Urinary output was monitored and fluid resuscitation with furosemide infusion was used if necessary. Laboratory assessment of coagulation parameters was performed daily and clotting abnormalities were corrected only in cases of active bleeding, or an invasive procedure. Specific antidotes and drug therapies such as silibinin[13] and corticosteroids were used if deemed necessary.

MARS treatment initiation criteria

The criteria for initiating MARS treatment and treatment protocols are summarized in Table 1. In some patients, MARS treatment was commenced in the absence of encephalopathy, particularly in ALF patients who had ingested a lethal amount of toxin or if laboratory parameters indicated progressive liver failure despite the best possible standard medical therapy. As a general rule, we treated only the first exacerbation of chronic alcoholic liver disease.

In the MARS apparatus, the flow rate was 150 mL/min in the blood and albumin circuit and 500 mL/min in the dialysis circuit with bicarbonate buffered dialysate. Ultrafiltration was adjusted to control intravascular volume balance. Anticoagulation was used if permitted by the coagulation status and platelet count of the patient; dalteparin or epoprostenol were used most often. A detailed description of the operational systems of the MARS machine can be found in our previous study[14].

Table 1 Indications for MARS and treatment protocols

Etiology	MARS treatment initiation criteria	Treatment protocol
ALF	Rapid deterioration of hepatic synthetic function and clinical condition despite standard medical therapy and (one of the following criteria)	Twenty-two hours sessions daily until the native liver recovers
	Ingestion of a lethal dose of a known hepatotoxin (mushroom, paracetamol, iron, *etc*)	A suitable transplant organ is found
	Patient fulfills the criteria for highly urgent Ltx	Irreversible organ damage occurs
AOCLF	Rapid deterioration of hepatic synthetic function and clinical condition despite standard medical therapy and (two of the following criteria)	Eight hours sessions based on the daily assessment of the surgeon and anesthesiologist until the patient's clinical condition improves
	Hyperbilirubinemia, bil > 400 μmol/L	A suitable transplant organ is found
	Hepatorenal syndrome type 1	Irreversible organ damage occurs
	Progressive hepatic encephalopathy (grade ⩾ 2)	
GF	No set criteria; depends on the assessment of the transplant surgeon and anesthesiologist	No set protocol; based on the daily assessment of the surgeon and anesthesiologist

ALF: Acute liver failure; AOCLF: Acute-on-chronic liver failure; GF: Graft failure.

Table 2 Demographic, clinical, and treatment data at the beginning of MARS treatment

Characteristic	All patients	ALF			AOCLF		Graft failure
		Toxic	Unknown cause	Other	Alcohol-related	Other	
Number of patients	192	63	41	9	45	17	11
Age, years	49 (14-81)	41 (14-81)	51 (19-68)	43 (32-58)	52 (30-71)	54 (16-75)	47 (18-62)
Sex, % male (*n*)	48 (93)	48 (30)	32 (13)	33 (3)	69 (31)	47 (8)	36 (4)
Body mass index, kg/m^2	26 (17-56)	24 (17-40)	28 (19-37)	28 (23-34)	27 (18-46)	27 (20-56)	27 (19-56)
MARS sessions/patient	2 (1-13)	2 (1-8)	3 (1-12)	3 (1-9)	2 (1-13)	2 (1-9)	2 (1-4)
Duration of MARS session, h	16.5 (4-22.5)	15.0 (5.5-22)	16.8 (4-22)	16.0 (6.4-22)	20.1 (7.8-22)	18.3 (4.5-22.5)	17.5 (9.5-22)
Mechanically ventilation used, % (*n*)	36 (69)	29 (18)	34 (14)	56 (5)	29 (13)	41 (7)	73 (8)
Vasoactive-medications used, % (*n*)	43 (82)	33 (21)	27 (11)	33 (3)	47 (21)	82 (14)	63 (7)
Renal insufficiency, % (*n*)	49 (94)	33 (21)	37 (15)	44 (4)	60 (27)	76 (13)	73 (8)
MELD score	32 (5-52)	27 (5-48)	32 (23-50)	27 (23-46)	39 (17-52)	36 (27-44)	26 (20-47)
Mean encephalopathy grade before treatment (± SD)	1.8 (1.5)	1.6 (1.6)	2.0 (1.4)	2.4 (1.7)	1.5 (1.4)	1.9 (1.6)	2.0 (1.9)
Mean encephalopathy grade after treatment (± SD)	1.4 (1.6)	1.4 (1.7)	1.5 (1.7)	1.4 (1.6)	1.1 (1.5)	1.9 (1.8)	1.3 (1.7)
P	< 0.001	NS	0.05	0.04	0.02	NS	0.059

All demographic values are expressed as median (range) or percentage of patients (number of patients). Encephalopathy grades are expressed as mean ± SD). NS: Non-significant.

Measurements and data collection

For all MARS-treated patients, detailed information regarding the patient and treatment session was collected prospectively on a specially designed data collection sheet. Demographic data and clinical parameters were recorded at the beginning and end of each treatment. Baseline measurement was performed at the beginning of the first MARS session. The endpoint was the end of the last MARS session, death, or LTX. At both time points, blood samples were analyzed for cell counts, coagulation factor levels, plasma levels of liver enzymes, bilirubin, ammonium ion, urea, creatinine, blood gases, and electrolytes. The value furthest from the normal range of each measured variable during treatment was not included in the present analysis. The model for end-stage liver disease (MELD) score was calculated according to the standard formula by the United Network for Organ Sharing (UNOS)[15-17] at ICU admission. Survival at 1-year and need for LTX were recorded.

Statistical analysis

All data were analyzed with SPSS for Windows version 15.0 (SPSS, Chicago, IL, USA). The Wilcoxon signed rank test was used for repeated scale measurements before and after treatment within groups. The Mann-Whitney *U* test was applied when scale measurements were compared between groups. The Pearson χ^2 and Fisher exact tests were used to compare outcomes and binominal results between groups. $P \leqslant 0.05$ was considered statistically significant.

Stepwise binary logistic regression analysis was performed to determine factors predicting survival in each etiological subgroup. Variables analyzed included all collected demographic, clinical and treatment-related variables (Table 2) and all laboratory parameters at baseline (Table 3). Missing laboratory values were replaced with the median value of that laboratory result in all patients. The median was used instead of the mean because of the skewed distribution of most results. Special attention was given to variables that changed during MARS treatment and parameters that differed between transplant-free survivors and non-survivors/transplanted patients. The odds ratio (OR) and 95% confidence interval (CI) for each predictive variable were calculated. The best combination

Table 3 Changes in laboratory parameters during MARS treatment in different liver failure subgroups

	Before MARS	After MARS	Percent change	*P*	Before MARS	After MARS	Percent change	*P*	Before MARS	After MARS	Percent change	*P*
	Toxic ALF (*n* = 63)				Unknown-cause ALF (*n* = 41)				Other ALF (*n* = 9)			
Hemoglobin g/L	110 (77-170)	100 (59-136)	-9	< 0.001	110 (74-146)	98 (71-134)	-11	0.001	98 (80-131)	88 (82-120)	-10	NS
Leucocytes 10^9/L	8.6 (1.0-29.2)	8.7 (2.4-33.9)	1	NS	8.9 (2.8-21.9)	8.4 (2.9-42.5)	-6	NS	7.6 (2.6-41)	11.4 (1.7-30.6)	50	NS
Platelets 10^9/L	130 (11-438)	80 (9-349)	-38	< 0.001	140 (48-511)	74 (25-327)	-47	< 0.001	97 (37-248)	85 (19-184)	-12	0.04
CRP g/L	9 (5-157)	15 (5-186)	67	< 0.001	8 (5-120)	10 (5-142)	25	0.006	30 (5-331)	33 (5-148)	10	NS
Creatinine μmol/L	79 (35-1318)	51 (17-585)	-35	< 0.001	84 (36-572)	54 (17-337)	-36	< 0.001	85 (57-567)	53 (23-149)	-38	0.02
Urea mmol/L	4.8 (0.8-31.2)	1.7 (0.2-11.7)	-65	< 0.001	6.3 (1.0-25.6)	1.8 (0.8-58)	-71	< 0.001	12.0 (4.2-29.3)	4.3 (1.0-6.7)	-64	0.01
NH_4-ion μmol/L	75 (18-512)	55 (3.5-258)	-26	< 0.001	75 (24-244)	56 (20-309)	-25	0.006	81 (8-317)	45 (17-176)	-45	NS
Bilirubin μmol/L	84 (4-761)	97 (6-355)	15	0.05	472 (35-725)	301 (10-570)	-36	< 0.001	372 (62-694)	190 (94-348)	-49	NS
AST U/L	842 (15-24360)	282 (15-5240)	-67	< 0.001	427 (50-18140)	183 (19-4080)	-57	< 0.001	600 (37-12640)	83 (43-2227)	-86	NS
ALT U/L	1120 (11-12500)	565 (5-9970)	-50	< 0.001	550 (71-11946)	174 (33-7790)	-68	< 0.001	217 (22-6710)	171 (21-1321)	-21	0.04
γ-GT U/L	72 (8-2139)	55 (9-1279)	-24	0.01	106 (20-503)	49 (5-238)	-54	< 0.001	157 (21-1422)	62 (22-1010)	-61	NS
FV %	33 (5-201)	51 (5-149)	55	0.07	33 (5-119)	23 (7-101)	-33	NS	55 (7-100)	53 (26-127)	-3	NS
AT3 %	44 (15-125)	41 (15-122)	-6	0.002	26 (15-78)	27 (15-68)	4	NS	32 (19-110)	33 (18-92)	3	NS
TT (%)	22 (6-80)	30 (6-112)	36	NS	17 (6-44)	18 (6-68)	3	NS	26.5 (6-53)	36 (16-52)	36	NS
INR	2.5 (1.1-7.7)	2 (1.0-9.9)	-20	NS	3.1 (1.5-9.9)	2.8 (1.5-10)	-10	NS	2.3 (1.4-5.5)	1.8 (1.4-3)	-22	NS
	Alcohol-related AOCLF (*n* = 45)				Other AOCLF (*n* = 17)				Graft failure (*n* = 11)			
Hemoglobin g/L	101 (59-129)	94 (75-123)	-7	0.01	96 (71-130)	91 (75-104)	-5	NS	96 (86-117)	99 (71-123)	3	NS
Leucocytes 10^9/L	17.2 (5-39.3)	16.7 (2.1-45.7)	-3	NS	10 (1.4-30.7)	5.7 (1.3-30)	-43	0.01	9.5 (1.7-14.9)	8.8 (2.1-20.8)	-7	NS
Platelets 10^9/L	129 (15-508)	83 (6-349)	-36	< 0.001	82 (27-238)	57 (19-215)	-30	0.004	81 (27-453)	83 (16-206)	2	NS
CRP g/L	32 (5-110)	38 (5-160)	19	NS	35 (5-67)	38 (5-110)	9	0.01	22 (6-172)	23 (9-64)	5	NS
Creatinine μmol/L	167 (49-686)	65 (20-216)	-61	< 0.001	210 (29-325)	52 (17-149)	-75	< 0.001	159 (39-301)	70 (22-121)	-56	0.003
Urea mmol/L	17.5 (1.8-56.5)	3.5 (0.7-18.3)	-80	< 0.001	16.9 (3.2-27.4)	3.1 (1.2-12.3)	-82	< 0.001	16.5 (6.0-32.3)	4.7 (1.3-10.0)	-72	0.004
NH_4-ion μmol/L	73 (12-311)	60 (23-144)	-18	0.05	89 (19-223)	62 (21-96)	-30	0.03	47 (17-177)	32 (25-69)	-32	NS
Bilirubin μmol/L	513 (17-840)	271 (14-499)	-47	< 0.001	481 (278-909)	283 (134-530)	-41	0.001	311 (107-720)	223 (74-348)	-28	0.009
AST U/L	156 (27-4540)	140 (13-1959)	-10	0.002	210 (31-1230)	144 (58-1222)	-31	0.03	321 (27-12560)	226 (53-87380)	-30	NS
ALT U/L	74 (9-2904)	69 (8-2480)	-7	< 0.001	100 (18-681)	85 (27-286)	-16	0.07	722 (38-9460)	4400 (48-25120)	509	NS
γ-GT U/L	198 (29-1086)	147 (19-810)	-26	< 0.001	69 (19-429)	61 (12-342)	-12	NS	180 (26-2385)	174 (34-1619)	-3	NS
FV %	53 (8-125)	46 (8-131)	-12	< 0.001	34 (7-124)	26 (8-124)	-24	0.005	79 (7-131)	77 (13-107)	-3	NS
AT3 %	34 (13-88)	29 (15-67)	-13	0.001	29 (15-100)	23 (15-100)	-21	0.01	54 (15-137)	56 (17-104)	-4	NS
TT (%)	22 (9-135)	17 (6-49)	-23	0.02	20 (6-96)	17 (6-73)	-15	NS	42 (8-139)	47 (20-113)	-12	NS
INR	2.4 (1.3-5.6)	2.9 (1.3-9.9)	21	0.03	2.4 (1-8.5)	2.9 (1.1-8.5)	21	0.01	1.5 (0.9-6.4)	1.5 (1.0-2.8)	0	NS

All laboratory values are expressed as median (range). NH_4-ion: Ammonium ion (normal range: 36-86 mmol/L); AST: Aspartate aminotransferase; γ-GT: gamma glutamyltransferase; FV: Coagulation factor Ⅴ (normal range: 80%-120%); AT3: Antithrombin Ⅲ (normal range 80%-120%); TT (%): Thrombin time (normal range, 70%-130%) (includes coagulation factors Ⅱ, Ⅶ, Ⅹ).

of significantly predictive variables was selected using the R^2 score and Hosmer-Lemeshow goodness of fit test.

RESULTS

Baseline characteristics of MARS-treated patients

Our study population consisted of 113 patients with ALF, 62 with AOCLF, 11 with GF, and six with miscellaneous liver failure (Figure 1). In total, 192 MARS treatment cases were included in this study. Four patients were treated before LTX and afterwards because of graft failure. These treatment sessions were categorized separately as individual cases, first according to the primary liver failure etiology, and later on as graft failure cases. In 30% (58/192) of all treated cases, alcohol was either partly (mixed intoxication, *n* = 13) or directly (chronic alcoholic-liver disease, *n* = 45; graft failure caused by alcohol, *n* = 1) related to liver failure. The median number of MARS treatments per patient was two (range: 1-13), and the median duration of one session was 16.5 h (range 4-22.5 h). Contraindications to LTX prior to MARS treatment were present in 35% (67/192) of cases and included substance abuse, serious psychiatric illness, patient decision, serious concomitant disease (e.g.

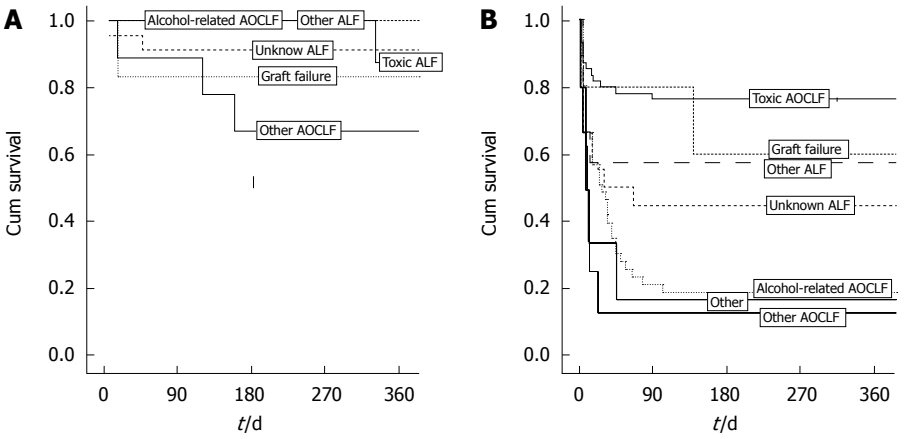

Figure 2 Kaplan-Meier cumulative survival for transplanted (A) and non-transplanted (B) MARS-treated patients with different causes of liver failure.

malignancy), and age > 80 years. In addition, 18 patients became untransplantable during MARS treatment: six because of serious, uncontrollable infections, and 12 because of multiorgan failure or brain death.

Etiologic subgroups differed significantly at baseline with respect to demographic data, clinical condition, and severity of liver failure (Table 2). The percentage of patients who required vasoactive medication and those with renal insufficiency were higher in the AOCLF and GF than in the ALF group. The highest median MELD scores were observed in association with alcohol-related AOCLF (39; range 17-52) and the lowest in association with ALF caused by toxicity (27; range 5-48) and GF (26; range 20-47) (Table 2). Encephalopathy grade decreased significantly in most subgroups during treatment. Changes in laboratory values during MARS treatment are presented in Table 3.

Outcome and characteristics of the subgroups

Kaplan-Meier 1-year survival curves for MARS-treated transplanted and non-transplanted patients are presented in Figure 2. The 1-year survival rate of all transplanted patients was 86% (43/50).

Patients with ALF: Patients with ALF were categorized into three subgroups according to etiology: toxic, unknown, and other (Figure 1). The toxic ALF subgroup was further subdivided into paracetamol-related and non-paracetamol-related intoxication caused by other drugs and toxins (e.g. *Amanita phalloides* or herbal products). The "other ALF" subgroup included patients with pregnancy-related ALF, Budd-Chiari syndrome, acute seropositive hepatitis, and hepatic trauma. A detailed analysis of the outcomes in these patients with ALF can be found in our previous study[18].

The 1-year overall survival rate of all ALF patients was 74% (84/113). The 1-year survival of transplanted ALF patients was 91% (30/33). The percentage of transplanted patients was 3% (1/32) of those with paracetamol-related ALF, 23% (7/31) of those with non-paracetamol-related ALF, 56% (23/41) of those with unknown-etiology ALF, and 22% (2/9) of those with other-etiology ALF. One-year survival rates of the transplanted and non-transplanted patients are shown in Figure 2. Six ALF patients died while waiting for a suitable graft. Half of these patients were non-encephalopathic at the initiation of MARS treatment.

Patients with AOCLF : All AOCLF patients were Child-Pugh class C and had a median MELD score of 36 (range 17-52). In 24% (11/45) of alcohol-related AOCLF and 12% (2/17) of other-etiology AOCLF patients, the liver was still enlarged and showed signs of steatosis.

In the alcohol-related AOCLF group, two abstinent patients received transplants. In other-etiology AOCLF, nine patients received transplants. The 1-year survival rates of non-transplanted and transplanted patients were, respectively, 19% (8/34) and 100% (2/2) in alcohol-related AOCLF, and 13% (1/8) and 67% (6/9) in other-etiology AOCLF.

In the alcohol-related AOCLF subgroup, the 1-year survival rate in non-transplanted patients with enlarged livers and signs of steatosis was significantly higher than in the other patients [55% (6/11) *vs* 6% (2/32); $P = 0.002$]. Both of these groups were comparable at baseline. The median MELD scores were 35 (range 24-48) in those with enlarged livers and steatosis and 39 (range 17-52) in the other patients.

Graft failure patients: In GF patients, 1-year survival rate was 73% (8/11). Four patients with early GF and two with late GF underwent retransplantation. The 1-year survival rates of non-retransplanted and retransplanted patients were, respectively, 50% (2/4) and 75% (3/4) in early GF, and 100% (1/1) and 100% (2/2) in late GF.

Miscellaneous etiology patients: All six patients in the subgroup with miscellaneous-etiology AOCLF had a contraindication to LTX. Only one patient with acute pancreatitis and ALF survived 1 year, and all other patients died within 2 mo of ICU admission.

Prognostic factors predicting 6-mo survival

The etiology of liver failure was highly significant in predicting patient outcome ($P < 0.0001$). The alcohol-related AOCLF subgroup with contraindications to LTX had the highest percentage of non-surviving patients.

In survival analysis, groups were divided into two categories: (1) transplant-free survivors, and (2) non-survivors and transplant recipients. At baseline, within the

Table 4 Demographic data, clinical condition, and laboratory parameters before treatment in survivors and non-survivors treated with MARS

	ALF			AOCLF		
	Transplant-free survivors	Non-survivors and transplant recipients	*P*	Transplant-free survivors	Non-survivors and transplant recipients	*P*
Demographic and clinical data at baseline						
Number of patients (*n*)	54	59		9	53	
Age, years (range)	39 (14-81)	50 (19-71)	< 0.0001	52 (30-58)	52 (16-75)	NS
Sex, male % (*n*)	52 (28)	31 (18)	0.021	67 (6)	62 (33)	NS
BMI, kg/m^2	24 (16.9-39.7)	27 (17-40)	NS	32 (18-38)	26 (20-56)	NS
MARS sessions/patient	2 (1-5)	3 (1-12)	< 0.0001	1 (1-3)	2 (1-13)	NS
Mechanical ventilation used % (*n*)	20 (11)	44 (26)	0.007	33 (3)	32 (17)	NS
Vasoactive-medication used % (*n*)	28 (15)	34 (20)	NS	33 (3)	60 (32)	NS
Renal insufficiency % (*n*)	32 (17)	39 (23)	NS	56 (5)	66 (35)	NS
MELD score (range)	24 (5-48)	32 (7-50)	< 0.0001	28 (17-48)	37 (25-52)	0.08
Encephalopathy grade prior to treatment	0 (0-4)	3 (0-4)	< 0.0001	1 (0-4)	1 (0-4)	NS
Laboratory values at baseline						
Hemoglobin g/L	113 (78-170)	106 (74-160)	NS	106 (80-127)	99 (59-130)	NS
Leucocytes 10^9/L	8.3 (1-23.4)	9.1 (2.6-41)	0.045	9.7 (1.4-35.3)	15.5 (1.6-39.3)	NS
Platelets 10^9/L	146 (11-351)	130 (37-511)	NS	131 (69-383)	107 (15-508)	NS
CRP g/L	10 (5-331)	8 (5-153)	NS	28 (8-58)	35 (5-110)	NS
Creatinine μmol/L	77 (35-1318)	91 (36-567)	NS	128 (56-556)	210 (29-686)	NS
Urea mmol/L	5.2 (1.1-31.2)	7.8 (0.8-29.3)	NS	8.4 (2.3-39.6)	17.45 (1.8-56.5)	NS
NH_4-ion μmol/L	50 (8-512)	99 (24-389)	< 0.0001	66 (12-241)	75 (19-311)	NS
Bilirubin μmol/L	71 (4-761)	425 (8-694)	< 0.0001	455 (17-745)	514 (143-909)	NS
AST U/L	732 (15-24360)	497 (50-20900)	NS	214 (27-2030)	164 (31-4540)	NS
ALT U/L	1165 (11-12500)	708 (71-10890)	NS	69 (9-2904)	87 (10-897)	NS
γ-GT U/L	61 (8-503)	109.5 (20-2139)	0.013	230 (44-398)	109 (19-1086)	NS
FV %	51 (7-201)	31 (5-101)	0.012	65 (41-124)	48 (7-125)	0.028
AT3 %	52 (15-125)	27 (15-110)	< 0.0001	40 (15-100)	29.5 (13-88)	0.043
TT (%)	26 (6-80)	16 (6-49)	< 0.0001	26 (9-96)	21 (6-135)	NS
INR	2.3 (1.1-7.7)	3.2 (1.4-9.9)	< 0.0001	2 (1-4.3)	2.4 (1-8.5)	NS
Albumin g/L	29.9 (19.2-46.4)	24.8 (11.5-41.2)	< 0.0001	23.3 (13.6-31.8)	21.8 (14.7-32.7)	NS

ALF group, the non-survivors and transplant recipients differed significantly from the transplant-free survivors in several clinical and laboratory parameters, including MELD score and levels of bilirubin, ammonium ion, and coagulation factors (Table 4).

In the AOCLF group, transplant-free survivors compared with non-survivors and transplant recipients had similar baseline values, except coagulation factor V and antithrombin Ⅲ plasma levels differed significantly (Table 4).

Factors predictive of survival were tested separately using stepwise binary logistic regression analyses in each etiological subgroup. The unwanted or negative endpoint was defined as death within 6 mo or LTX. All demographic and clinical parameters and laboratory values before MARS treatment presented in Tables 2 and 3 were included in these analyses. Additionally, in the toxic etiology subgroup, the analysis of the causative drug or poison was taken into account as an independent prognostic factor.

In the paracetamol-related toxic ALF subgroup, the only significant predictor of survival was the grade of hepatic encephalopathy at the beginning of treatment (OR, 0.345; 95% CI, 0.154-0.774; $P = 0.001$). Based on the equation below, hepatic encephalopathy grades from 0 to 4 predicted the probability (p) of survival at 6 mo to be, respectively, 98%, 94%, 85%, 65% and 40%. The positive predictive and negative predictive values, and the overall predictive accuracy based on the equation and the data were 92%, 57% and 84%, respectively. The sensitivity and the specificity were 67% and 89%, respectively.

$p = 100 \times [1/(1 + e^{-(3.831\text{-HE}*1.064)})]$.

In the non-paracetamol-related toxic ALF subgroup, significant predictors of survival were thrombin time, (TT) (OR, 1.103; 95% CI, 1.000-1.217; $P = 0.049$), and hepatic encephalopathy grade at the beginning of treatment (OR 0.562; 95% CI, 0.305-1.035; $P = 0.064$). The predicted probability (p) of survival at 6 mo was approximated by inserting the patient's variables into the equation given below. For example, a TT of 21% and encephalopathy grade of 2 predicted a survival probability of 45%. The positive predictive and negative predictive values and the overall predictive accuracy based on the equation and the data were 76%, 79% and 77%, respectively. The sensitivity and the specificity were 73% and 81%, respectively.

$p = 100 \times [1/(1 + e^{-(1.120 + \text{TT}*0.098\text{-HE}*0.577)})]$.

In the unknown etiology ALF subgroup, significant predictive factors for survival were coagulation factor V levels (OR, 1.052; 95% CI, 1.007-1.099; $P = 0.02$) and alanine aminotransferase ALT plasma levels (OR, 1.001; 95% CI, 1.000-1.001; $P = 0.013$). The predicted probability (p) of survival at 6 mo was approximated by inserting the patient's ALT and factor V levels (FV) into the equation given below. For example, an ALT value of 550 U/L and FV value of 33% gave a 6-mo survival probability of 6.5%. The positive and negative predictive

values and the overall predictive accuracy based on the equation and the data were 60%, 86% and 83%, respectively. Referring to the data, the sensitivity and the specificity of the equation were 94% and 38%, respectively.

$p = 100 \times [1/(1 + e^{-(-4.894 + ALT*0.001 + FV*0.051)})]$.

We were unable to find significant predictive variables in other etiological subgroups.

DISCUSSION

To the best of our knowledge, the present study of 188 patients represents the largest number of MARS-treated patients with liver failure investigated thus far in a single treatment unit. This is also believed to be the first attempt to examine prognostic factors predicting survival in different etiological subgroups of MARS-treated patients with liver failure. Prognostic and treatment efficacy estimations are becoming increasingly more important in today's ICU management, as the number of patients and per-patient costs continue to increase. In 2001, when we began using MARS therapy, it was unclear which patients would benefit from the treatment. The only available data on MARS at the time were from a few small studies conducted on patients with AOCLF[19-24]. Therefore, data with the planned protocol were collected prospectively from every MARS-treated patient in our ICU.

In AOCLF, some randomized studies[5,6,8,22] and small case series of MARS-treated patients[25-27] have reported favorable effects. However, conflicting reports have also emerged[28]. In review articles, MARS has been considered an effective and safe treatment[29,30], although in an early meta-analysis, it did not significantly reduce mortality[31]. In contrast to these studies, ours did not reveal any beneficial effect of MARS treatment on the outcome of AOCLF, except as bridging therapy. One reason that might explain this difference is patient selection. Our criteria for initiation of MARS therapy included at least two of the following: hyperbilirubinemia, hepatorenal syndrome, and encephalopathy. In the aforementioned studies, enrolled patients were in better clinical condition prior to treatment, which makes a direct comparison of results challenging. Also, in most other studies, follow-up time was significantly shorter than 1 year.

In the present study, we found that in the subgroup of non-transplanted patients with alcohol-related AOCLF, Child-Pugh class C, and no signs of hepatic steatosis or enlargement, the mortality was very high (94%). This suggests that MARS treatment in these patients was not beneficial, as it did not seem to improve the final outcome. Recently, a study by Wolff *et al*[32] led to a similar conclusion. Considering the poor survival results in patients with alcohol-related AOCLF, one might argue that MARS treatment should have been commenced earlier in the course of the disease, to benefit the patient. The optimal timing of MARS treatment in AOCLF was, and still is, unknown and requires further investigation. As most of our patients with AOCLF had end-stage cirrhosis, the regenerative capacity of the native liver was probably non-existent and the benefit of MARS treatment was only in bridging the patient to LTX. Furthermore, we were able to find a subgroup of patients with alcohol-related AOCLF with significantly better survival: patients with enlarged livers and signs of steatosis seemed to benefit more from MARS treatment, and had a significantly higher transplantation-free survival rate, even though all other baseline laboratory and clinical values were similar to those in other cirrhotic patients.

The 1-year survival rate of all transplanted patients was high (86%) in our study. Particularly in transplanted patients with ALF, the overall 1-year survival rate of 91% was significantly higher than the 1-year survival rates of 67%-83% that have been reported by western transplantation registers[15,33,34] and studies[35,36] in the past decade. This finding might be attributable to the observed improvement in many clinical and laboratory parameters in patients with AOCLF or ALF during MARS treatment. Additionally, the grade of encephalopathy decreased significantly in most patients. The fact that these patients were, therefore, in better clinical condition prior to LTX might contribute to the high overall survival of transplanted patients. The favorable effect of MARS treatment on laboratory parameters, as we observed, has also been reported in many small, uncontrolled studies[26,27,37-44]. However, the improvement in laboratory values alone might be only temporary and does not necessarily predict a favorable outcome. However, as noted in other studies, MARS treatment also seems to stabilize the patient hemodynamically and prevent the worsening of encephalopathy[18,19,41,45,46], thus helping to bridge the patient successfully to LTX.

The main goal of our study was to identify prognostic factors that could predict survival and help in the selection of patients for MARS treatment. Based on our data, we built mathematical prediction models to estimate the 6-mo survival probability of MARS-treated patients. The most important factor for survival and spontaneous recovery was the etiology of the liver disease. In both toxic ALF subgroups, the grade of encephalopathy prior to MARS treatment was a prognostic factor, and in the subgroup of non-paracetamol-related ALF, coagulation factor levels were prognostic as well. In the subgroup of unknown-etiology ALF, ALT levels and coagulation factor V levels were prognostic, but surprisingly, encephalopathy grade was not. In other liver failure subgroups, we were unable to detect variables that would accurately predict survival. MELD score was not included in this analysis because the target of this study was not to compare outcomes to previously investigated prognostic criteria, such as early lactate[47], the Clichy criteria[48], and the King's College criteria[49] for non-MARS treated patients with ALF, and the Child-Pugh class[50,51] or MELD score[15,52-54] for patients with AOCLF.

Thrombin time and factor V activity level were significant predictive factors in patients with non-paracetamol-related and unknown-etiology ALF, respectively. These factors emerged as predictive despite the fact that, in our ICU, treatment is usually started with intensive replacement of coagulation factors, to enable

the safe placement of a large-bore dialysis catheter. At the measurement of all baseline laboratory variables, the replacement therapy had already been administered to most patients. Additionally, calculation of the MELD score necessitates the use of the international normalized ratio (INR), and therefore, most patients scored much lower than they would have without prior coagulative therapy. Our results concurred with previous studies that factor V level[48,55] and prothrombin time[49] are significant predictors of survival in patients with ALF. Also, in our predictive model for unknown-etiology ALF, high plasma levels of ALT (which is released into the bloodstream from injured hepatocytes) correlated with improved survival. High serum ALT levels might reflect the initial stage of acute liver injury. As the condition progresses, there is less liver tissue to be destroyed, and thus the ALT levels fall, and the liver's capacity for spontaneous recovery and the probability of transplantation-free survival diminish. The simultaneous plunge in factor V levels further reflects the declining synthetic capacity of the remaining liver mass.

In the present study, the grade of hepatic encephalopathy at the beginning of treatment was a predictive factor of survival in the toxic-etiology ALF subgroup. In these patients, treatment was initiated in the absence of encephalopathy if the patient had ingested a lethal amount of toxin, such as *Amanita* mushrooms. This early treatment might improve the prognosis of these patients. Still, despite ICU and MARS treatment, three originally non-encephalopathic patients with ALF died while waiting for a suitable graft. This finding further emphasizes the importance of early referral and prompt commencement of treatment in a specialized unit[56,57]. In previous studies with non-MARS-treated patients with ALF, encephalopathy grade[49] as well as other clinical, serological and physical variables have been reported as predictors of survival[48,49,55,58-62].

Yuan *et al*[63] have reported recently on a study of the prognostic factors for early (30-d) mortality in MARS-treated patients scheduled for LTX. The study included a heterogeneous group of 50 patients with liver failure regarded and analyzed as one group. In Yuan's study, 68% of patients were transplanted compared to 25% of our patients. The 30-d postoperative survival was 82% in transplanted and 50% in non-transplanted patients. These 30-d survival figures correspond remarkably well with our respective 1-year outcome results (86% survival for transplanted and 47% survival for non-transplanted patients). The prognostic factors that correlated with early postoperative mortality in Yuan's study were sequential organ failure (SOFA) score, creatinine, INR, tumor necrosis factor-α, and interleukin-10. Encephalopathy grade was not considered significant in this analysis[63].

One of the limitations of our study is that it represents a very specific population and distribution of patients with liver failure in Finland. As the etiological factors and causative agents behind ALF and AOCLF vary between countries, the applicability of our results to other scenarios is probably reduced. In addition, there is also a likely selection bias associated with the acceptance of patients with AOCLF for MARS treatment. In Finland, alcohol-related AOCLF is a fairly common condition; these patients are usually treated in basic medical wards and not referred to our unit because chronic alcohol abuse with diagnosed cirrhosis is usually considered a contraindication to ICU treatment. Furthermore, the specificity and especially sensitivity of our predictive models were far from optimal. Ideally, a good prognostic tool would accurately, easily and cheaply predict the patient's survival probability and the need for LTX in the very early stages of the disease. In the real world, however, prognostic calculations can never predict the fate of an individual patient with 100% accuracy, as there are always exceptions to the rule, special circumstances, and multiple factors that were not considered in the prognostic model. At best, such calculations can be used as aids and facilitators, but not as substitutes, for the physician's clinical assessment.

In conclusion, the present study showed that, despite ICU and MARS treatment, patients with AOCLF and end-stage cirrhosis do not seem to benefit from MARS treatment without the possibility of LTX. In patients with alcohol-related AOCLF, we now use MARS treatment only with those whose liver is still enlarged and steatotic, with recovery capacity. The grade of encephalopathy and levels of coagulation factors were not consistently significant prognostic factors in all liver failure groups treated with MARS.

COMMENTS

Background

Rapidly failing liver function is a medical emergency that carries a high risk of mortality. Molecular adsorbent recirculating system (MARS) treatment is an extracorporeal albumin dialysis apparatus that has been used in the treatment of liver failure to enable native liver regeneration or as a bridge to liver transplantation (LTX).

Research frontiers

The impact of MARS treatment on outcome as well as clinical and laboratory variables has been investigated widely in small non-randomized studies. However, prognostic factors predicting survival in MARS-treated patients have only been explored in one study so far. The current hotspot of the research is to determine which patient groups actually benefit from MARS treatment. Another interesting question is whether there are patient groups that do not gain from MARS and should not be treated.

Innovations and breakthroughs

The prognostic factors predicting survival in MARS-treated patients have only been explored in one previous study by Yuan *et al*. That study comprised 50 patients with a heterogeneous etiological background and a follow-up of 30 d. The present study contained 188 patients with a heterogeneous etiological distribution. However, prognostic factors were searched for with logistic regression analysis in each etiological subgroup independently. In addition the follow-up time was 1 year. In the present study, the etiology of liver failure was the most important predictor of survival. In acute liver failure (ALF) with toxic etiology (e.g. paracetamol), the grade of encephalopathy before MARS treatment was a significant prognostic factor. In ALF of unknown etiology, coagulation factor V and liver enzyme alanine aminotransferase levels were prognostic. According to the authors results, the MARS treatment of a cirrhotic patient with an acute-on-chronic liver failure (AOCLF) is not meaningful in terms of prognosis if the patient is not eligible for transplantation.

Applications

MARS treatment appears to be a safe and effective treatment in ALF patients and those chronic liver disease patients who are eligible for LTX.

Terminology

MARS is an extracorporeal albumin dialysis machine that removes water-

soluble and albumin-bound toxins from the patient's blood. ALF is defined as rapid deterioration of liver synthetic and metabolic function in a person with no previous liver disease. AOCLF is a condition in which a previously stable patient with chronic liver disease experiences a rapid deterioration of liver function caused by some triggering event (e.g. gastrointestinal bleeding, infection or ingestion of alcohol). Cirrhosis is a consequence and the histological hallmark of chronic liver disease. It is characterized by the replacement of normal liver cells by scar tissue and eventually it leads to liver failure. Hepatic encephalopathy is a potentially reversible neuropsychiatric disorder presenting as a decreased level of consciousness associated with liver failure

Peer review

This is a well-written paper which has high clinical relevance. This was a unique single-center study of a large population of patients with ALF or AOCLF.

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Lower baseline ALT cut-off values and HBV DNA levels better differentiate HBeAg(-) chronic hepatitis B patients from inactive chronic carriers

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Abstract

AIM: To determine whether new cut-off values for alanine aminotransferase (ALT) and baseline hepatitis B virus (HBV) DNA levels better differentiate HBeAg(-) chronic hepatitis B (CHB) patients from inactive chronic carriers.

METHODS: Ninety-one patients [32 HBeAg(+) CHB, 19 inactive carriers and 40 HBeAg(-) CHB] were followed up for 2 years and were tested for HBV DNA levels by a PCR-based assay. ALT was tested twice during the last 6 mo using new cut-off values: ULN (upper limit of normal) 30 IU/L for males, 19 IU/L for females. Diagnostic accuracy, sensitivity, specificity, positive and negative predictive values were calculated by discriminant analysis.

RESULTS: When using the revised ALT cut-off values, the lowest optimal HBV DNA level that differentiated HBeAg(-) CHB patients from inactive carriers was 50 000 copies/mL. The diagnostic accuracy of HBV DNA to determine inactive carriers with a cut-off of 50 000 copies/mL was similar to the previously recommended cut-off of 100 000 copies/mL (91%). HBV DNA levels were lower than the cut-off value in 95% of inactive carriers and in 28% of HBeAg(-) CHB patients. With ALT < 30 IU/L in men and < 19 IU/L in women and HBV DNA levels < 100 000 copies/mL, the risk of CHB is 5%. On the other hand, if ALT values were > 30 IU in men and > 19 IU in women and baseline HBV DNA levels were > 100 000 copies/mL, the risk is 86%.

CONCLUSION: New cut-off values for ALT together with HBV DNA levels proposed by AASLD (American Association for the Study of Liver Diseases) and NIH (National Institute of Health) consensus seem appropriate to characterize inactive carriers.

Key words: Alanine aminotransferase; Chronic hepatitis B; Hepatitis B antigens; Viral DNA

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INTRODUCTION

In the natural history of chronic hepatitis B virus (HBV) infection, sero-conversion from HBeAg(+) to HBeAg(-) and anti-HBe antibody (+) leads to low HBV replication and to normalization of aminotransferases[1]. Such changes have long been considered a reliable clinical indicator of passage to an inactive and innocent state of chronic hepatitis B[1]. However, 2 clinical forms of HBeAg(-) chronic hepatitis B exist after sero-conversion. The first form is the "inactive carrier state" which comprises an absence of HBeAg, a lack of symptoms, persistently normal alanine aminotransferase (ALT) and low or undetectable HBV DNA (< 100 000 copies/mL) levels. The second form is described as "HBeAg(-) chronic hepatitis B", and comprises an absence of HBeAg, presence of symptoms, elevated ALT and a high HBV DNA

level (> 100 000 copies/mL). Differentiation between these 2 forms of chronic hepatitis B is difficult when HBV DNA levels are between 10 000 and 100 000 copies/mL and the distinction depends on the sequential determination of ALT activity[2]. Indeed, patients with HBeAg(-) chronic hepatitis B demonstrate wild fluctuations in serum ALT and 20%-30% of these patients with histologically documented chronic hepatitis have normal ALT at the time of presentation[3]. Consequently, patients with HBeAg(-) chronic hepatitis B with normal liver enzymes may be misdiagnosed as being in an inactive chronic carrier state and thus be denied treatment by mistake.

ALT is the most commonly used enzyme in the evaluation of liver disease. Recently, it has been suggested that the upper normal limit (ULN) for ALT should be decreased to 30 IU/L for men and 19 IU/L for women[4]. Minimal increases in serum ALT levels, although within the classic normal range, have also been reported to be significantly associated with increased risk of liver-related mortality in the general population[5]. Moreover, a recent study showed that chronic hepatitis B patients with normal serum ALT levels were also at risk of development of cirrhosis and hepatocellular carcinoma[4,6]. Therefore, chronic hepatitis B patients with normal ALT levels may be at risk of progressive liver disease. The determination of a more reliable cut-off value for ALT activity is very important.

PCR assays allow the detection of very low serum HBV DNA levels (100 copies/mL)[6], and are more powerful tests than hybridization techniques[7]. An arbitrary serum HBV DNA level of 100 000 copies/mL has been proposed by the United States National Institute of Health (NIH) workshop[8] to differentiate HBeAg(-) chronic hepatitis B from the inactive carrier state. However, a study from France found that 98% of inactive carriers had HBV DNA levels < 100 000 copies/mL at presentation and 97% of patients had HBV DNA levels persistently below 100 000 copies/mL during a 1-6 year follow-up. A study from Greece reported that a cut-off value of 100 000 copies/mL would lead to misclassification of 13% of their patients with HBeAg(-) chronic hepatitis B and possible denial of treatment. The researchers suggested a HBV DNA cut-off level of 30 000 copies/mL to be more appropriate for differentiating the inactive carrier state from HBeAg(-) chronic hepatitis B[9,10]. A study from the United States found that no HBV DNA cut-off value existed for differentiating inactive carriers from patients with HBeAg(-) chronic hepatitis B[11]. Thus, the appropriate HBV DNA value for differentiating inactive chronic carriers from patients with HBeAg(-) chronic hepatitis B remains to be determined.

HBV DNA levels at baseline have been associated with an increased risk of cirrhosis and hepatocellular carcinoma[12-14]. Moreover, Yuen *et al*[13] reported an increased risk of complications as well as increased mortality from liver disease in patients with a prolonged low level of viremia (10 000-100 000 copies/mL). An important question is whether the use of new cut-off values for ALT (30 U/L in men and 19 U/L in women) and baseline HBV DNA levels better differentiates HBeAg(-) chronic hepatitis B patients from inactive chronic carriers. In the current study, we evaluated whether the combination of the revised cut-off values for ALT and the baseline HBV DNA levels correctly predicted the classification of patients with HBeAg(-) chronic hepatitis B.

MATERIALS AND METHODS

One hundred and ninety patients with HBeAg(-) chronic hepatitis B infection were recruited and studied retrospectively. Inclusion criteria were: HBsAg(+), HBeAg(-) for at least 6 mo. Patients with fatty liver, alcohol use > 30 g/d, obesity (BMI > 28), hepatocellular carcinoma, hepatitis C, hepatitis D and HIV viral co-infection were excluded. Patients with decompensated liver disease including bilirubin level > 1.5 mg/dL (25.6 μmol/L), prothrombin time > 15 s or INR > 1.7, albumin level < 3.4 g/dL, ascites, bleeding esophageal varices or hepatic encephalopathy were also excluded. 59 patients were enrolled. Thirty two patients with HBeAg(+) chronic hepatitis B were added to the study population for comparison. All patients with HBeAg(-) chronic HBV infection had baseline ALT determined at the first visit. During follow-up (12-24 mo), all patients had serum ALT determined at 3 and 6 mo intervals and underwent liver biopsy in cases of increased ALT activity at least twice (ULN for ALT values 40/30 were used). Patients were classified into HBeAg(-) chronic hepatitis B if they had increased ALT activity, HBV DNA > 100 000 copies/mL and histological findings compatible with chronic hepatitis. On the other hand, patients with HBeAg(-) chronic hepatitis B infections were classified into the inactive chronic carriers if they had persistently normal ALT values at the first visit and through follow-up, and HBV DNA < 100 000 copies/mL. Patients with normal ALT values were followed up with ALT measured every 3-6 mo for the first 2 years and every 12 mo thereafter. No patient received antiviral or immunosuppressive therapy during the study period. Standard biochemistry was performed by Olympics analyzer (Hamburg, Germany). ULN for ALT were: 40 U/L in men and 30 U/L in women for the old normal range and 30 U/L in men and 19 U/L in women for the new normal range[7]. Patients with increased ALT and HBV DNA < 100 000 copies/mL (n = 7) and histological findings compatible with chronic hepatitis were incorporated into the HBeAg(-) chronic hepatitis B group. The rare cases with persistently normal ALT, and HBV DNA > 100 000 copies/mL (n = 4) were not classified because of the very small number of patients. All serum samples were processed in the same laboratory using the same methods and the same reference values. Virology tests, including HBsAg, HBeAg, anti-HBe antibody, anti-HBS antibody, and anti-HB core antibody, anti-HDV antibody, anti-HCV antibody, and anti-HIV antibody, were evaluated by commercially available enzyme immunoassays. Serum HBV DNA was measured by a sensitive quantitative PCR assay (Amplicor HBV monitor test, Roche Diagnostic Systems, Branchburg, NJ)

Table 1 Baseline characteristics of 91 patients with chronic hepatitis B virus infection

Patient characteristics	HBeAg (+) Chronic hepatitis B *n* = 32	HBeAg(-) hepatitis B		[1]*P*
		Inactive carriers *n* = 19	HBeAg(-) chronic hepatitis *n* = 40	
Gender (M/F)	23/9	17/2	30/10	0.001
Age	32 ± 11	39 ± 10	39 ± 10	0.010
BMI	24.9 ± 2.5	25.1 ± 2.6	25.9 ± 2.0	0.300
Histological grade	4.6 ± 1.9 (4-12)	ND	4.4 ± 1.2 (4-18)	0.500
Histological stage	1.3 ± 1.4 (0-6)	ND	1.1 ± 1.3 (0-6)	0.500
ALT (IU/L)	118 (16-190)	24 (16-30)	52 (20-116)	0.001
AST (IU/L)	71 (11-142)	26 (16-34)	38 (17-143)	0.001
HBV DNA (median, copies/mL)	1.9×10^{11}	4150	2×10^{5}	0.001

Values are median, or mean ± SD. [1]Comparison between 3 groups. ND: Not done. Every 19 IU/mL of HBV DNA equals 100 copies/mL. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; HBV: Hepatitis B virus.

with sensitivity levels of 200 copies/mL[15]. An arbitrary value of 100 copies/mL was assigned to samples with undetectable HBV DNA for statistical comparison. Liver histology for inflammation and fibrosis stage was performed according the classification of Ishak[16].

Statistical analysis

Statistical analysis was performed using the Winstat program. Results are presented as median (range) or mean ± SD. The Mann-Whitney test and Kruskal-Wallis test for non-parametric data were used for comparison between 2 and among 3 groups, respectively. The Spearman test was used for the correlation between 2 quantitative variables. The diagnostic validity of a single baseline measurement of serum ALT, aspartate aminotransferase (AST), and HBV DNA levels, and the validity of the combined new normal range for ALT and HBV DNA levels were tested regarding correct classification of HBeAg(-) into those with HBeAg(-) chronic hepatitis B or those who were inactive carriers. Cut-off levels for ALT were ULN values. Cut-off levels for HBV DNA, were 5000, 50 000 and 100 000 copies/mL respectively[15,16]. The percentage of cases correctly classified by each diagnostic test as well as sensitivity, specificity, and positive and negative predictive values were calculated by discriminant analysis. The likelihood ratio was calculated according to the formula (LR+ = sensitivity/1-specificity), LR- = (1-sensitivity/specificity). In all cases, tests of significance were two-tailed, with a significance level less than 0.05. The study was approved by an institutional ethics committee and each patient signed an informed consent form.

RESULTS

Patients with HBeAg(-) chronic hepatitis B were older than the HBeAg(+) patients (39 ± 10 *vs* 32 ± 11, $P < 0.001$). Men were predominant in all groups. The 91 patients with chronic HBV infection were classified as: 32 HBeAg(+) patients, 19 who were inactive chronic carriers and 40 patients determined to have HBeAg(-) chronic hepatitis B. Baseline ALT values were within the normal range according to the new cut-off values (30 U/L in men and 19 U/L in women) in 24 patients (40%) and were increased in 60% of the 59 patients with HBeAg(-) chronic hepatitis B infection. Demographic, histological and laboratory characteristics are presented in Table 1. Baseline ALT levels were lower in patients with HBeAg(-) than HBeAg(+) chronic hepatitis B (median 32 U/L, mean 52 ± 29.8 U/L *vs* median 52 U/L, mean 118 ± 44.9 U/L, $P < 0.01$). There was no difference in inflammatory grade (4.4 ± 1.2 *vs* 4.6 ± 1.9, $P < 0.5$) or fibrosis stage (1.1 ± 1.3 *vs* 1.3 ± 1.4, $P < 0.5$) between HBeAg(-) and HBeAg(+) chronic hepatitis B patients. No cases of bridging fibrosis or early cirrhosis were documented.

Median HBV DNA levels were lowest in the inactive chronic carriers, intermediate in the HBeAg(-) chronic hepatitis B patients, and highest in HBeAg(+) chronic hepatitis B patients ($P < 0.001$, Table 1). Serum HBV DNA was less than 50 000 copies/mL in 95% of inactive chronic carriers and undetectable (< 200 copies/mL) in 20% of them.

A baseline serum HBV DNA cut-off level of 50 000 copies/mL could correctly classify 91% of HBeAg(-) patients, achieving better classification than baseline traditional ALT (40 U/L in men and 30 U/L in women) and AST enzyme cut-off levels. The diagnostic accuracy of the HBV DNA cut-off of 50 000 was similar to the commonly proposed serum HBV DNA cut-off of 100 000 copies/mL, but much better than the latest recent proposition of 5000 copies/mL or 1000 copies/mL (Table 2). The cut-off value of 50 000 copies/mL also performed better than 5000 copies/mL in the subgroup of patients with the new ALT cut-off values (30 U/L in men and 19 U/L in women), achieving correct classification in 91% of cases. When using ALT × 1.3 combined with HBV DNA cut-off of 50 000 copies/mL, we increased the diagnostic accuracy from 80% to 85% (Table 3).

A serum HBV DNA cut-off level at 50 000 copies/mL alone had better sensitivity, specificity, and positive or negative predictive values for discrimination between patients with HBeAg(-) chronic hepatitis B and inactive chronic carriers as compared with any other single variable [HBV DNA with a cut-off at 5000 copies/mL, ALT and AST level (Table 2)]. Multivariate discriminant analysis showed that all single variables could classify our patients into HBeAg(-) chronic hepatitis B and inactive carrier state, but the standardized canonical discriminant function co-efficiency was higher for serum HBV DNA with a cut-

Table 2 Validity of ALT, AST and serum HBV DNA levels for the differentiation of patients with HBeAg(-) chronic hepatitis B from inactive chronic HBsAg carriers

Laboratory test	SP %	SS %	PPV %	NPV %	Diagnostic accuracy %	Inactive carriers *n* = 19	HBeAg(-) hepatitis B *n* = 40
ALT (> *vs* ≤ 2 ULN)	100	33	100	41	54	0/19	27/13
ALT (> *vs* ≤ 1.3 ULN)	47	90	78	69	78	10/9	36/4
AST > ULN	84	45	86	42	58	3/16	22/18
HBV DNA (> *vs* < 50000 copies/mL)	57	73	97	62	80	1/18	29/11
HBV DNA 50000 + ALT > (1.3 ULN)	95	80	96	69	85	1/18	32/8

Every 19 IU/mL of HBV DNA equals 100 copies/mL. Upper normal limit (ULN) for ALT: 30 IU/L for men and 19 IU/L for women.

Table 3 Validity of HBV DNA levels for the differentiation of patients with HBeAg(-) chronic hepatitis B from inactive carriers and normal ALT values

HBV DNA levels (Copies/mL)	Specificity %	Sensitivity %	Predictive value %		Correct classification	Inactive carriers	HBeAg(-) hepatitis B
			PPV	NPV			
HBV DNA > *vs* < 50000	95	78	78	95	91	1/18	4/1
HBV DNA > *vs* < 100000	95	78	78	95	91	1/18	4/1
HBV DNA > *vs* < 20000	73	80	44	93	75	5/14	4/1
HBV DNA > *vs* < 5000	57	75	78	52	70	8/11	3/2

HBV DNA: Every 19 IU/mL equals 100 copies/mL.

Table 4 Validity of ALT (ULN 30/19) and baseline serum HBV DNA levels for the differentiation of patients with HBeAg(-) chronic hepatitis B from inactive carriers

Laboratory test	SP %	SS %	PPV %	NPV %	Diagnostic accuracy %	Inactive carriers *n* = 19	HBeAg(-) hepatitis B *n* = 40
ALT (> *vs* ≤ 30 M/19 F)	100	92	100	86	95	0/19	37/3
HBV DNA (> *vs* ≤ 100000 copies/mL)	73	72	85	56	72	5/14	29/11
ALT+ HBV DNA	100	92	100	86	95	0/19	37/3

off at 50000 copies/mL ($f = 0.76$) than for serum with a cut-off 5000 ($f = 0.69$) or ALT level ($f = 0.44$). Using cross validation in univariate discriminant analysis, if the cut-off was set at 50000 copies/mL, serum HBV DNA could correctly classify 80% of the patients with HBeAg(-) chronic hepatitis B infection, and could correctly classify 85% if the cut-off was set at 50000 copies/mL combined with ALT × 1.3 above the new ULN (30 U/L in men and 19 U/L in women). ALT and AST could correctly classify only 78% and 58% of cases, respectively (Table 2). Of the 5 patients with HBeAg(-) chronic hepatitis B and HBV DNA cut-off levels < 50000 copies/mL, 4 patients (95%) were inactive chronic carriers. All inactive carrier patients had normal baseline AST as well as normal new ALT values. Similarly, all patients with chronic hepatitis B with normal ALT levels initially, also had normal AST values initially. A serum HBV DNA level at 50000 copies/mL could correctly classify 91% of these cases similar to the correct classification of 91% achieved by HBV DNA of 100000 copies/mL, and more than the 75% achieved by HBV DNA < 20000 copies/mL and the 70% by HBV DNA < 5000 copies/mL (Table 2). If ALT values were > 30 U/L in men and > 19 U/L in women and baseline HBV DNA levels were > 100000 copies/mL, the likelihood (odds) of having HBeAg(-) chronic hepatitis B is raised by 16 relative to the previous probability of disease, with a diagnostic accuracy of 95%, a negative predictive value of 86%, a positive predictive value of 100%, a sensitivity of 92%, and a specificity of 100% (Table 4).

Within the HBeAg(-) chronic hepatitis B group, patients with elevated baseline ALT (> 30 U/L in men and 19 U/L in women) had a significantly higher median serum HBV DNA level compared with those patients with normal baseline ALT values (median 96053 copies/mL, mean 870000 copies/mL *vs* median 16202 copies/mL, mean 370000 copies/mL, $P < 0.01$). There was less overlap in HBV DNA levels between HBeAg(-) chronic hepatitis B patients and inactive carriers when the new normal range for ALT was used. Serum HBV DNA levels did not correlate with age, gender or histology but correlated well with the new ALT levels in all patient populations ($r = 0.42$, $P < 0.001$) and to a lesser extent ($r = 0.3$, $P < 0.01$) in patients with HBeAg(-) chronic hepatitis B (Figure 1).

DISCUSSION

New medical treatment has proven effective for patients with chronic hepatitis B; therefore early and definitive

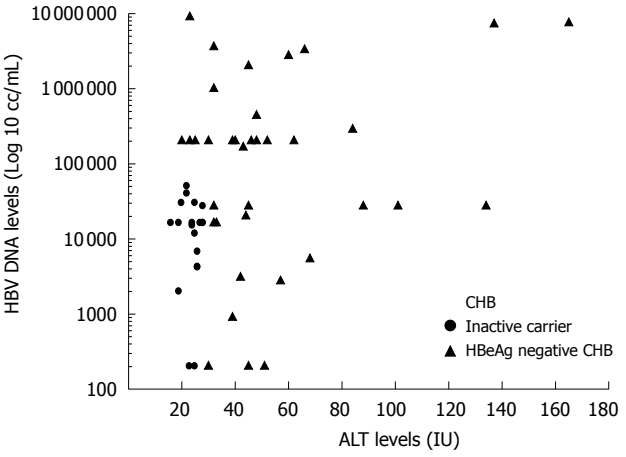

Figure 1 Correlation between ALT values and HBV DNA serum levels in inactive carriers and in HBeAg(-) chronic hepatitis B (r = 0.3, P < 0.01). CHB: Chronic hepatitis B. Every 19 IU/mL of HBV DNA equals 100 copies/mL.

detection is crucial[3]. Decreasing the ULN values for ALT levels significantly increases the ability to detect HBeAg(-) chronic hepatitis B. The results of the present study indicate that if ALT values are < 30 U/L in men and < 19 U/L in women with baseline HBV DNA levels < 100 000 copies/mL, the likelihood of being diagnosed with HBeAg(-) chronic hepatitis B is 5%. On the other hand, if ALT values are > 30 U/L in men and > 19 U/L in women with HBV DNA levels > 100 000 copies/mL, the likelihood of HBeAg(-) chronic hepatitis B diagnosis is 86%. The results indicate also that HBV DNA cut-off levels of 100 000 copies/mL as proposed by the NIH workshop[8] to characterize inactive carriers seems appropriate, and that HBV DNA cut-off levels lower than 50 000 copies/mL do not add to the diagnostic accuracy of HBeAg(-) chronic hepatitis B.

In HBeAg(-) chronic hepatitis B, ALT levels can flare with an intervening period of normal values, can continue to increase without flare, or demonstrate intermittent flares superimposed on a continuous elevation[17,18]. The majority of our patients belonged to the first profile. The current study is in keeping with the work of Martinot-Peignoux *et al*[9] who showed that HBV DNA levels remained stable with a median of 1000-10 000 copies/mL in 85 inactive carriers followed for 1-6 years, and only 2% had HBV DNA levels > 100 000 copies/mL, supporting the NIH recommendation. However, the Martinot-Peignoux study did not include patients with HBeAg(-) chronic hepatitis B and the proportion of patients with HBV DNA < 50 000 copies/mL was not reported, thus a direct comparison with our findings cannot be made. Moreover, compared with the Martinot-Peignoux study, a stricter definition of inactive chronic carrier was used in the current study, using the new normal range for ALT (30 U/L in men and 19 U/L in women) and lower HBV DNA levels. Reports from Greece found that 13% of 134 patients with HBeAg(-) chronic hepatitis B had serum HBV DNA levels < 100 000 copies/mL, indicating that a cut-off of 100 000 copies/mL could lead to misclassification of these patients and possible denial of treatment[19]. The authors suggested that a cut-off HBV DNA levels of 30 000 copies/mL might be more appropriate for differentiating inactive HBsAg carriers from patients with HBeAg(-) chronic hepatitis B. The Greek study used old values for normal ALT levels (40 U/L in men and 30 U/L in women) and was based on serum HBV DNA levels taken at admission. In the present study, levels < 50 000 copies/mL did not add to the diagnostic accuracy of the classification of HBeAg(-) chronic hepatitis B. The current work is also in agreement with the finding of Chu *et al*[11] that HBV DNA values above 100 000 copies/mL would exclude 95% of inactive carriers but also 22% of HBeAg(-) chronic hepatitis patients if testing of ALT was performed at admission and again after 6 mo. More recently, Degertekin & Lok[20] concluded that a cut-off 5000 copies/mL is more appropriate for differentiating inactive carriers from HBeAg(-) chronic hepatitis B patients. Our study contrasts with the Degertekin & Lok study in that HBV DNA cut-off levels of 5000 copies/mL did not improve diagnostic accuracy for differentiating HBeAg(-) chronic hepatitis patients from inactive carriers. Given the variable natural history of chronic hepatitis B viral infection and variable genotype and mutations, it is possible that this threshold level might differ from one population to another and may vary with time depending on the host immune status and other exogenous factors[21].

HBV viremia was detected in the vast majority of patients with HBeAg(-) chronic hepatitis B, and the 50 000 copies/mL cut-off also performed better than 5000 copies/mL in the subgroup of patients with new normal ALT levels, achieving correct classification in 91% of cases[22]. Using ALT × 1.3 above the new cut-off limit combined with a HBV DNA cut-off of 50 000 copies/mL increased the diagnostic accuracy from 80% to 85%. With this threshold level, it is possible to identify individuals with very low risk of progressive liver disease, in whom current treatment offers no benefit and who may require less frequent monitoring. This depends on host factors such as CD4 immune response, viral factors such as HBV genotypes and mutation in the core promoter and precore regions and environmental factors such as alcohol consumption[21].

More than 90% of our subjects belonged to the ethnic Druze religious group and were infected > 40 years ago. Thus our data may not be applicable to individuals with adult-acquired HBV infection or with perinatal acquired HBV infection but who are younger than 40 years old[23]. Long term longitudinal studies of inactive carriers have reported that 15%-24% developed HBeAg(-) chronic hepatitis and 20%-30% had moderate to severe inflammation while up to 20% had advanced fibrosis or cirrhosis[24-26]. Moreover, *post hoc* analysis of phase Ⅲ clinical trials of entecavir have confirmed that patients with < 2 ULN of the old ALT values (40 U/L in men and 30 U/L in women) at pretreatment were less likely to undergo HBeAg seroconversion or to have detectable serum HBV DNA[22].

Our results do not confirm previously reported data that single AST measurement is better than serum HBV DNA or ALT levels for the differentiation between HBeAg(-) patients with active and inactive liver dis-

ease[26]. In contrast, we found that that the new baseline cut-off for ALT levels (30 U/L in men and 19 U/L in women) clearly performs better than AST in achieving correct classification of HBeAg(-) chronic hepatitis B, 78% and 58%, respectively (Table 2). ALT, a biochemical marker of inflammation, showed a greater increase in HBeAg(+) patients when compared with HBeAg(-) chronic hepatitis B patients, suggesting that HBeAg has immunomodulatory action[27]. There was no correlation between serum HBV DNA and histological grade in either HBeAg(-) or HBeAg(+) chronic hepatitis B patients, in agreement with previous reports of HBV DNA levels and histological severity in HBeAg(-) chronic hepatitis B patients[28]. In addition, we found no correlation between HBV DNA levels and the pattern of histological inflammation (portal, periportal necrosis, and interlobular or confluent necrosis).

Limitations of our study are: (1) lack of genotype sequencing in order to identify HBV mutants; however reports on the relationship between precore and core promoter variants, serum HBV DNA levels and liver diseases are inconclusive[29-31]; (2) HBV DNA levels may vary widely with time and any classification of HBeAg(-) chronic hepatitis B may subsequently change. Therefore, longitudinal evaluation of HBV DNA levels was not performed in the current study[31]; (3) the small number of patients; (4) a short period of follow up; (5) the absence of histology in the healthy carrier group.

In conclusion, with new cut-off values for ALT (30 U/L in men and 19 U/L in women), there may be less overlap in HBV DNA levels between inactive carriers and HBeAg(-) chronic hepatitis B patients. The HBV DNA cut-off levels of 100 000 copies/mL proposed by the NIH workshop to characterize inactive carriers, is accurate[8]. HBV DNA cut-off levels < 50 000 copies/mL do not improve diagnostic accuracy for differentiating between HBeAg(-) chronic hepatitis B patients from inactive carriers. Longer follow-up and repeated determination of HBV DNA and ALT serum levels are required to definitively exclude HBeAg(-) chronic hepatitis B and to classify a patient into the inactive carrier state.

COMMENTS

Background

Two clinical forms of HBeAg(-) chronic hepatitis B exist after hepatitis Be antigen seroconversion. The first form is the "inactive carrier state" which comprises absence of HBeAg, a lack of symptoms, persistently normal alanine aminotransferase (ALT), and low or undetectable hepatitis B virus (HBV) DNA (< 100 000 copies/mL) levels. The second form is described as "HBeAg(-) chronic hepatitis B", and includes the absence of HBeAg, the presence of symptoms, elevated ALT and high HBV DNA levels (> 100 000 copies/mL). Differentiation between these 2 forms of chronic hepatitis B is difficult when HBV DNA levels are between 10 000 and 100 000 copies/mL and the distinction depends on the sequential determination of ALT activity. Consequently, patients with HBeAg(-) chronic hepatitis B with normal liver enzymes according to the old ALT values (ALT 40 IU/L for men and 30 IU/L for women) may be misdiagnosed as inactive chronic carriers.

Innovations and breakthroughs

The study indicates that lower baseline ALT cut-off values (ALT 30 U/L in men, 19 U/L in women) in combination with a baseline HBV DNA level (> 100 000 copies/mL) better differentiate HBeAg(-) chronic hepatitis B patients from inactive chronic carriers.

Applications

The clinical application is that many patients misdiagnosed as inactive carriers may now benefit from antiviral treatment.

Peer review

The study is interesting from a clinical point of view and that the authors studied different cut-off values for baseline HBV DNA and ALT levels to better classify HBeAg(-) subjects into chronic hepatitis patients and inactive chronic carriers.

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BRIEF ARTICLES

Improving quality of colonoscopy by adding simethicone to sodium phosphate bowel preparation

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Abstract

AIM: To evaluate the effectiveness of simethicone in enhancing visibility and efficacy during colonoscopy.

METHODS: A prospective, double-blind, randomized, placebo-controlled study was conducted. One hundred and twenty-four patients were allocated to receive 2 doses of sodium phosphate plus 240 mg of tablet simethicone or placebo as bowel preparation. Visibility was blindly assessed for the amount of air bubbles and adequacy of colon preparation. Total colonoscopic time, side effects of the medication, endoscopist and patient satisfaction were also compared.

RESULTS: Sodium phosphate plus simethicone, compared to sodium phosphate plus placebo, improved visibility by diminishing air bubbles (100.00% *vs* 42.37%, $P < 0.0001$) but simethicone failed to demonstrate improvement in adequacy of colon preparation (90.16% *vs* 81.36%, $P = 0.17$). Endoscopist and patient satisfaction were increased significantly in the simethicone group. However, there was no difference in the total duration of colonoscopy and side effects of the medication.

CONCLUSION: The addition of simethicone is of benefit for colonoscopic bowel preparation by diminishing air bubbles, which results in enhanced visibility. Endoscopist and patient satisfaction is also increased.

Key words: Simethicone; Colonoscopy; Bowel preparation

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INTRODUCTION

Colonoscopy is considered to be the gold standard investigation for assessing colonic lesions; but many factors, such as the quality of bowel preparation, endoscopist, and patient factors, may affect the diagnostic accuracy and therapeutic safety[1-5]. Inadequate bowel preparation has been reported in 10%-75% of colonoscopic examinations[1,3]. The ideal preparation for colonoscopy should be safe, acceptable to patients with negligible discomfort, and it should take effect on rapid cleansing[2-4]. Unfortunately, none of the preparations meets all of the requirements[2-4]. Several studies have evaluated the efficacy and side effects of regimens for bowel preparation[3-14]. In 2006, three medical organizations (the American Society for Gastrointestinal Endoscopy, the American Society of Colon and Rectal Surgeons, and the Society of American Gastrointestinal and Endoscopic Surgeons) suggested that polyethyleneglycol (PEG) should be a gold standard for colonoscopic bowel preparation (Grade IA), and aqueous sodium phosphate (NaP) was an alternative regimen to PEG solution (Grade IA)[2]. This consensus also stated that adjunctive therapy, such as bisacodyl, metoclopramide, and simethicone, was shown to improve the quality of bowel preparation[2].

Simethicone is an oral antifoaming agent that reduces bloating, abdominal discomfort, and abdominal pain by promoting the clearance of excessive gas along the gastrointestinal tract[15]. Chemically, simethicone is a mixture of polydimethylsiloxanes that works by reducing the surface tension of air bubbles and causing the coalescence of small bubbles into larger ones that pass more easily with belching or flatulence[15]. Simethicone is not absorbed into the bloodstream and is, therefore, considered relatively safe[15]. Its use prior to diagnostic procedures such as gastroscopy[16], transabdominal ultrasound[17,18], anorectal ultrasound[19], computed tomography scan[20] and capsule endoscopy has been increasingly reported[21,22].

The presence of air bubbles along the colonic surface can prevent the clear visualization of the whole colon. Simethicone works as an adjunct to bowel preparation with the purpose of diminishing foam formation and improving visualization during colonoscopy[23-28]. However, most of the previous studies that demonstrated enhanced quality of bowel preparation used PEG for the bowel preparation regimen[23-28]. Furthermore, only liquid simethicone was used as an adjunct therapy in these studies[23-28]. The benefit of simethicone in improving colonic bowel preparation, however, was not explored in previous studies[23-28]. Moreover, other factors such as time of colonoscopy, endoscopist, and patient satisfaction have never been mentioned[23-28]. Although some endoscopists already use simethicone prior to performing colonoscopic examination in daily practice, the adjunctive use of simethicone in the standard bowel preparation regimen has not been uniformly accepted so far. We aimed to evaluate the beneficial effect of oral simethicone on bowel preparation, as compared to NaP alone, with regard to the degree of visibility and the quality of bowel preparation. We selected NaP solution as a bowel preparation regimen because of its tolerability. Based upon three medical consensuses, NaP solution is suggested to be an alternative bowel preparation with equal potency to PEG solution[2]. The addition of oral simethicone to the bowel preparation regimen before colonoscopy is more practical and more convenient than on-demand simethicone spraying due to time savings and prompt, clear visualization. Furthermore, the addition of simethicone may reduce the total colonoscopic time. The primary endpoint of this study was to compare the efficacy of simethicone to placebo in terms of the amount of air bubbles remaining and bowel preparation quality. The success rate and duration of colonoscopy, endoscopist satisfaction, and patient acceptability were the secondary endpoints.

MATERIALS AND METHODS

Protocol

A double-blind, randomized, placebo-controlled study was conducted between January 1, 2007 and December 31, 2007. The inclusion and exclusion criteria were shown in Table 1. All patients were instructed to consume a low-residual liquid diet one day prior to the date of the procedure. At the beginning of the study, they were allocated to receive 2 doses of either 45 mL of NaP plus 240 mg of tablet simethicone or 45 mL of NaP plus identically appearing placebo the evening before and the morning of the day of the procedure. During colonoscopy, we used meperidine and midazolam as the sedation regimen. Colonoscopic examinations were performed by 8 investigators (5 staff and 3 fellows). The details of the colonoscopic findings were recorded on DVDs. Endoscopic visibility was assessed for the amount of air bubbles and the adequacy of colon preparation by a single investigator who was blind as to the types and details of bowel preparation. Five areas of the colon (rectosigmoid, descending, transverse, ascending, and cecum) were graded for the amount of air bubbles. The amount of intraluminal air bubbles was classified into four grades as shown below[24]: Grade 0 = No or minimal scattered bubbles; Grade 1 = Bubbles covering at least half the luminal diameter; Grade 2 = Bubbles covering the circumference of the lumen; Grade 3 = Bubbles filling the entire lumen.

Table 1 Inclusion and exclusion criteria

Inclusion criteria
Adults aged 18-70 years of age scheduled for colonoscopy at the gastroenterological unit
Exclusion criteria
Renal insufficiency (Cr ⩾ 2 mg/dL)
Uncontrolled congestive heart failure (NYHC Ⅲ-Ⅳ)
Massive ascites
Myocardial infarction within 6 mo
Pregnancy
Coagulopathy
History of colonic surgery
Colonic obstruction
History of anti-flatulence and/or other laxative agent use within 1 wk
Refusal to participate in the study

The re-defined grading was classified by following the more practical report by Sudduth *et al*[24]. The most frequent grading was selected to represent the overall grading; for example, if the grade was 0, 0, 0, 1, 1 the patient was assigned an overall grade of 0. If there were several equal grades, the grading that was closest to the final grading would be selected. For example, when the grading was 0, 0, 1, 1, and 2, grade 1 was selected. Grades 0 and 1 were re-defined as the diminishing of air bubbles; grade 2 and 3 were re-defined as the failure to diminish air bubbles[24]. The adequacy of colon preparation was graded as follows[28]: Excellent = Small amounts of clear liquid; Good = Residual liquid stool, all mucosa seen; Adequate = Some particulate matter, > 90% of mucosa seen; Poor = Substantial particulate matter or solid stool, < 90% of mucosa seen; Unacceptable = Solid stool throughout the colon.

Excellent, good, and adequate were grouped and re-defined as acceptable for adequacy of colon preparation; poor and unacceptable were grouped and re-defined as unacceptable for adequacy of colon preparation. The

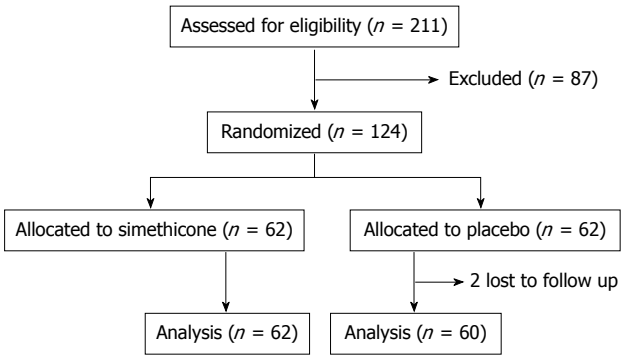

Figure 1 Patient disposition.

success rate, total duration of colonoscopy, side effects of medication, endoscopist satisfaction, and patient satisfaction were compared between the two groups. Endoscopist satisfaction was evaluated for air bubbles and adequacy of colon preparation by a self-rated questionnaire with a 4-degree scale ranging from very poor to very good[10]. The side effects of the bowel preparation regimens were recorded. Patient satisfaction was scored with a Visual Analog Scale, ranging from 0-10, where 0 represented "very poor" and 10 represented "excellent" [10]. The study was registered in the national clinical trials database (ClinicalTrials.gov identifier NCT00615303) and was approved by the Hospital Ethics Committee. The study was conducted according to the Helsinki Declaration guidelines.

Statistical analysis

The sample size was calculated based on the result of a previous study that revealed that simethicone improved colonic visibility by decreasing air bubbles (97.0% *vs* 75.0%). The calculated sample size of each group was 59 patients. Mean (SD) or median (range) was used to describe continuous data. Frequency (%) was used to describe categorical data. Independent *t* tests (or Mann-Whitney test) were used to compare the continuous characteristics and outcomes of interest data. Chi-square test (or exact test) was used to compare the categorical characteristics and outcomes of interest data. All analyses were performed using STATA version 9.0. $P < 0.05$ was accepted as statistically significant.

RESULTS

Two hundred and eleven patients were initially scheduled for colonoscopy. Eighty-seven patients were excluded because of failure to meet the inclusion criteria (10 patients did not give consent to the study, 38 patients with renal insufficiency received PEG instead of NaP solution, 12 patients had massive ascites, 8 patients had coagulopathy, 14 patients had a history of colonic surgery, and 5 patients had a history of colonic obstruction) (Figure 1). One hundred and twenty-four patients were recruited to the study. Two patients in the placebo group were lost to follow-up. Clinical characteristics of the study patients are shown in Table 2. There were no significant differences between the two groups. Mean age of both groups together was 57 years, and the proportion of male to female was 50 (41.0%): 72 (59.0%). The colonoscopic examination was not completed in 2 patients (1 from each group). Seventy-one (58.2%) and 51 (41.8%) patients underwent colonoscopy due to the presence of gastrointestinal symptoms and for screening purposes, respectively. The details of the endoscopic findings did not differ between the simethicone and placebo groups (Table 3).

NaP plus simethicone improved endoscopic visibility significantly by diminishing air bubbles more than NaP plus placebo, both when each segment of the colon was

Table 2 Baseline characteristics of the study groups (%)

Characteristics	Simethicone *n* = 62	Placebo *n* = 60
Age (yr); mean (SD)	57.5 (9.9)	56.5 (11.7)
Gender		
Male	27 (43.5)	23 (37.1)
Female	35 (56.6)	37 (61.7)
Indication for colonoscopy		
Screening	25 (40.3)	26 (43.3)
Symptomatic	37 (59.7)	34 (56.7)
Underlying diseases		
Diabetic	14 (22.6)	12 (20.0)
Hypertension	17 (27.4)	16 (26.7)
Coronary artery disease	2 (3.2)	3 (5.0)
Liver disease	10 (16.1)	5 (8.3)
Malignancy	5 (8.1)	8 (13.3)
GI disease	7 (11.3)	3 (5.0)
No underlying disease	18 (29.0)	22 (36.7)
Medication		
Antihypertensive agent	19 (30.7)	20 (33.3)
Hypoglycemic agent	12 (19.4)	11 (18.3)
Antiplatelet	4 (6.5)	7 (11.7)
No medication	25 (40.3)	27 (45.0)

Table 3 Colonoscopic results, endoscopic visibility and procedure time between the study groups (%)

	Simethicone *n* = 61	Placebo *n* = 59
Endoscopic findings		
Normal	17 (27.4)	16 (26.7)
Polyps	31 (50.0)	28 (46.7)
Cancer	1 (1.6)	3 (5)
Colitis	4 (6.5)	7 (11.7)
Nonspecific	2 (3.2)	2 (3.3)
Others	7 (11.3)	4 (6.7)
Degree of air bubbles		
Acceptable	61 (100.0)	25 (42.4)[b]
Cecum	60 (98.4)	38 (64.4)[b]
Ascending colon	59 (96.7)	32 (54.2)[b]
Transverse colon	60 (98.4)	30 (50.8)[b]
Descending colon	59 (96.7)	30 (50.8)[b]
Rectosigmoid colon	61 (100.0)	46 (78.0)[b]
Unacceptable	0	34 (57.6)
Adequacy of colon preparation		
Acceptable	55 (90.2)	48 (81.4)
Unacceptable	6 (9.8)	11 (18.6)
Duration of colonoscopy (min); mean (SD)	25.1 (13.2)	27.3 (13.0)

[b]$P < 0.0001$.

Table 4 Endoscopist and patient satisfaction for bowel preparation (%)

	Simethicone *n* = 61	Placebo *n* = 59
Endoscopist satisfaction		
Air bubble	48 (79.0)	19 (32.8)[a]
Adequacy of colon preparation	42 (68.9)	33 (55.9)
Patient satisfaction; mean (SD)	8.7 (1.8)	7.6 (1.9)[b]

[a]$P < 0.0001$, [b]$P = 0.002$.

considered separately and also when the colon was analyzed as a whole (100.0% *vs* 42.4%, $P < 0.0001$) (Table 3). Nevertheless, simethicone failed to decrease the amount of residual fecal material (90.2% *vs* 81.4%, $P = 0.17$) (Table 3). Endoscopist and patient satisfaction in simethicone group was higher than that in the placebo group (79.0% *vs* 32.8%, $P < 0.0001$ and 8.7 ± 1.8 *vs* 7.6 ± 1.9, $P = 0.002$) (Table 4). However, there was no difference in the total duration of colonoscopic examination between the simethicone and placebo groups (25.1 ± 13.1 *vs* 27.3 ± 12.9 min, $P = 0.27$). The issue of who performed the procedure did not affect the study result. Adverse drug reactions such as nausea and vomiting, abdominal pain, fatigue, and sleep disturbance were not significantly different between the two groups. Nausea and vomiting was found to be the most common side effect in this study (Table 5).

Table 5 Side effects of bowel preparation (%)

Symptoms	Simethicone *n* = 40	Placebo *n* = 43
Nausea and vomiting	12 (30.0)	16 (37.2)
Abdominal pain	5 (12.5)	6 (14.0)
Fatigue	2 (5.0)	1 (2.3)
Sleep disturbance	0	1 (2.3)

DISCUSSION

Colonoscopy is one of the most accurate investigations for colorectal screening and for assessing colonic lesions in patients who present with gastrointestinal symptoms such as hematochezia, diarrhea, or constipation. The quality of colonoscopy depends on multiple factors such as the redundancy of the colon, patient discomfort, and the type of bowel cleansing regimen. Inadequate bowel cleansing can impair visualization and colonic lesions. It may also prolong the duration of colonoscopy and increase patient discomfort. Previous studies were done to evaluate the efficacy of various bowel preparation regimens; however, the ideal bowel preparation regimen has not yet been found. PEG and NaP have been incorporated in the standard recommendations as bowel preparation for colonoscopy[2,3].

Simethicone is one of the adjunct therapies that can improve the quality of bowel preparation[23-28]. In this study, we report that the addition of simethicone to NaP is superior to the standard colonic bowel preparation with NaP alone, in terms of diminishing air bubbles and increasing patient acceptance to bowel preparation regimen. The endoscopic visibility was acceptable in 100.0% of patients in the simethicone group while it was found in only 42.4% in the placebo group. However, simethicone failed to raise the quality of colon preparation to a satisfactory level. Previous studies showed that simethicone improved the visibility of colonoscopy[23-28]. Sudduth *et al*[24] evaluated the efficacy of simethicone and NaP in 86 patients. The study revealed that simethicone improved colonic visibility by decreasing air bubbles (97.0% *vs* 75.0%, $P < 0.05$). Shaver *et al*[23] assessed the benefit of adding simethicone to Golytely in 120 patients. The study showed that simethicone decreased colonic foam (100.0% *vs* 67.0%, $P < 0.005$) and residual stool (5.3% *vs* 38.9%, $P < 0.05$)[23]. Compared to previous studies, we found more air bubbles along the gastrointestinal tract without clear explanation. Using NaP solution as a bowel preparation regimen instead of PEG may be one of the factors related to this. The addition of simethicone to bowel preparation did not yield any benefit in terms of bowel preparation adequacy.

The improvement in patient satisfaction with the bowel preparation regimen, which was never evaluated in previous papers, was reported here; although it was statistically significant, it may not be clinically relevant. Improving patient satisfaction in the simethicone group may be due to the fact that simethicone reduces gas and abdominal discomfort during colonoscopy. Patient satisfaction may encourage patient willingness to undergo repeated colonoscopy in the future. Simethicone is not absorbed into the bloodstream, and is therefore considered relatively safe with very few reports of bloating, constipation, diarrhea, gas, and heartburn[15]. In this study, the number of other side effects (e.g. nausea and vomiting, abdominal pain, fatigue and sleep disturbance) were equally distributed in both groups. These side effects may also be caused by NaP solution. Although simethicone improved visibility by decreasing air bubbles, we did not find any decrease in the total duration of colonoscopy. Moreover, the completion rate of colonoscopy between the two groups was not different. Patient factors (e.g. the length and redundancy of the colon), requirement of additional procedures (e.g. polypectomy and mucosal biopsy), and experience of the endoscopist are possible confounding factors that may lengthen the colonoscopic time.

The results of our study have some limitations. Firstly, there was no improvement of adequacy of colon preparation, and residual fecal materials were still present after the addition of simethicone. Secondly, we did not investigate other clinically important endpoints such as the reduction of missed lesions or interval neoplasm because there may be other factors, such as endoscopist factors, colonoscopic withdrawal time, and bowel preparation regimen that could affect the result of the study. Larger studies with different protocol designs are needed to answer these questions.

Air bubble reduction results in markedly enhanced visibility and, possibly, improvement in the quality of the colonoscopy. Endoscopist and patient satisfaction is also increased in the simethicone group. Thus, simethicone may be considered to be an adjunct therapy to NaP bowel preparation regimen in clinical practice.

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COMMENTS

Background

Colonoscopy is considered to be the gold standard investigation for colonic lesions. Sodium phosphate (NaP) is one of the bowel preparation regimens for colonoscopy; however, air bubbles can impair visibility during the examination.

Research frontiers

Simethicone is an oral antifoaming agent. The presence of air bubbles along the colonic surface can impede the clear visualization of the whole colon. This article aims to evaluate the effectiveness of simethicone in enhancing visibility and efficacy during colonoscopy.

Innovations and breakthroughs

The use of simethicone as an adjunct to the bowel preparation regimen has been studied and the quality of the bowel preparation was found to be increased. However, all of the previous studies used PEG plus simethicone as a bowel preparation regimen and only liquid simethicone was selected as an adjunct therapy. Furthermore, a decrease in haziness was still questionable. Colonoscopy duration and the satisfaction of the endoscopist and the patient have never been explored.

Applications

The addition of simethicone to NaP solution is of benefit for colonoscopic bowel preparation by diminishing air bubbles, resulting in enhanced visibility. Endoscopist and patient satisfaction is also enhanced after simethicone addition. Thus, simethicone should be considered as an adjunct to NaP solution for bowel preparation.

Peer review

This manuscript entitled "Improving Quality of Colonoscopy by adding Simethicone to Sodium Phosphate Bowel Preparation" shows outcomes of a prospective randomized control trial for additional benefit of simethicone for colon preparation. This study is well constructed and the study protocol itself is quite acceptable, but the outcomes show only a modest benefit of simethicone.

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BRIEF ARTICLES

Acute extensive portal and mesenteric venous thrombosis after splenectomy: Treated by interventional thrombolysis with transjugular approach

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Abstract

AIM: To present a series of cases with symptomatic acute extensive portal vein (PV) and superior mesenteric vein (SMV) thrombosis after splenectomy treated by transjugular intrahepatic approach catheter-directed thrombolysis.

METHODS: A total of 6 patients with acute extensive PV-SMV thrombosis after splenectomy were treated by transjugular approach catheter-directed thrombolysis. The mean age of the patients was 41.2 years. After access to the portal system *via* the transjugular approach, pigtail catheter fragmentation of clots, local urokinase injection, and manual aspiration thrombectomy were used for the initial treatment of PV-SMV thrombosis, followed by continuous thrombolytic therapy *via* an indwelling infusion catheter in the SMV, which was performed for three to six days. Adequate anticoagulation was given during treatment, throughout hospitalization, and after discharge.

RESULTS: Technical success was achieved in all 6 patients. Clinical improvement was seen in these patients within 12-24 h of the procedure. No complications were observed. The 6 patients were discharged 6-14 d (8 ± 2.5 d) after admission. The mean duration of follow-up after hospital discharge was 40 ± 16.5 mo. Ultrasound and contrast-enhanced computed tomography confirmed patency of the PV and SMV, and no recurrent episodes of PV-SMV thrombosis developed during the follow-up period.

CONCLUSION: Catheter-directed thrombolysis *via* transjugular intrahepatic access is a safe and effective therapy for the management of patients with symptomatic acute extensive PV-SMV thrombosis.

Key words: Mechanical thrombectomy; Portal vein; Splenectomy; Superior mesenteric vein; Thrombolysis; Thrombosis

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INTRODUCTION

Portal vein (PV) and superior mesenteric vein (SMV) thrombosis is an uncommon but lethal complication occurring after splenectomy[1,2]. The incidence of this type of complication ranges from 1.6% to 11% in some series[3] and from 6.3% to 10% in others[4,5]. Although many patients with PV and SMV thrombosis may be asymptomatic, the consequences of these thromboses can be severe, and include mesenteric ischemia and variceal bleeding, with a mortality rate of 5%-37%[2]. There are no uniform protocols for the

effective treatment of PV-SMV thrombosis following splenectomy, including duration of anticoagulation therapy and the potential effectiveness of prophylactic perioperative antiplatelet agents[6-9]. However, to avoid lethal complications, appropriate treatment should be performed as soon as possible, especially in patients with SMV involvement[4,6].

Recently, endovascular interventional techniques have been recognized as promising alternatives for the treatment of PV-SMV thrombosis. Case reports of the successful treatment of PV-SMV thrombosis include intra-arterial infusion of thrombolytics *via* the superior mesenteric artery (SMA) by the transfemoral artery[10,11], thrombolysis *via* a transjugular intrahepatic approach[12-14], and percutaneous transhepatic mechanical or pharmacologic thrombolysis[15-17]. The aim of the present study is to report the clinical outcome of 6 patients with acute symptomatic PV and SMV thrombosis after splenectomy who were treated with transjugular intrahepatic portal access aspiration thrombectomy and catheter-directed thrombolysis at our hospital.

MATERIALS AND METHODS

The study was approved by the institutional review board at our hospital. The potential risks and benefits of the procedure were explained, and informed consent was obtained from each patient.

Patients

Between March 2001 and October 2007, using the transjugular intrahepatic approach, we treated 6 patients (1 woman, 5 men) with a mean age of 41.2 years (range 32-52 years) who had symptomatic extensive PV and SMV thrombosis after open splenectomy. All 6 patients had acute abdominal symptoms of less than 3 wk (range, 3-16 d; average, 8.5 d).

Of these patients, four underwent splenectomy due to liver cirrhosis with portal hypertension, gastroesophageal variceal bleeding, and hypersplenism; splenectomy was performed in two patients due to portal hypertension and gastroesophageal variceal bleeding, caused by splenic vein occlusion. These 2 patients had a history of pancreatitis. All 6 patients had marked splenomegaly. The pre-splenectomy platelet count was less than $100 \times 10^3/mm^3$ ($3.5\text{-}6.5 \times 10^3/mm^3$; reference range, $100\text{-}300 \times 10^3/mm^3$), and in the normal range in 2 patients. The interval between the onset of symptoms of PV-SMV thrombosis and splenectomy was 11 to 35 d (median, 20.5 d).

All 6 patients initially presented with abdominal pain of insidious onset associated with nausea; 3 had distension, 3 had diarrhea, 2 had low-grade fevers, 1 had vomiting, and 1 had heme-positive stools. All patients were hemodynamically stable, and no clinical signs of peritonitis were noted at abdominal examination (Table 1).

Diagnostic evaluation

On admission, increased platelet count ($340\text{-}660 \times 10^3/mm^3$) was observed in 4 patients (Table 1). Increased white blood cell count ($12.5 \pm 1.5 \times 10^3/mm^3$; range, $11.5\text{-}14.0 \times 10^3/mm^3$) was found in 3 patients. Increases in transaminases (AST, 60-80 IU, reference range, 5-40 IU; ALT, 65-110 IU, reference range, 5-40 IU) were seen in 3 patients. Normal values were detected for thrombin time, serum D-dimer, C-reactive protein, serum D-lactate, and amylase. A workup for hypercoagulable states (factor V leiden, protein C and S deficiency, antithrombin Ⅲ deficiency, and lupus anticoagulant) yielded normal results.

Ultrasonography (US) and computed tomography (CT) were performed in all 6 patients. All patients had SMV thrombosis extending into the main portal vein which was confirmed by the imaging study.

General management

All patients were treated initially with bowel rest and nasogastric suction, intravenous fluid administration, broad-spectrum prophylactic antibiotics (including ampicillin, gentamycin, and metronidazole), and intravenous heparin adjusted to maintain the activated partial thromboplastin time ratio between 2.0 and 2.5 times the control.

Systemic anticoagulation after the diagnosis of PV and SMV thrombosis was assessed for 2 d in 4 cases and 3 d in 2 cases, however, the symptoms continued in 4 patients with worsening abdominal pain in 2 patients. After discussions with the surgery and medicine departments, and given the lack of clinical and radiographic suspicion for ischemic bowel, these patients were referred to the interventional radiology department for catheter-directed thrombolysis to achieve rapid restoration of PV-SMV flow. The mean time from admission to our institute to treatment with catheter-directed thrombolysis was 3.5 d, with a range of 2.5-5 d.

Indications and contraindications to interventional radiological thrombolysis

In our hospital, catheter-directed thrombolysis was employed in patients with acute and subacute PV and SMV thrombosis, with severe symptoms, and with persistent symptoms or worsening of symptoms despite anticoagulation.

Contraindications to interventional thrombolysis included mesenteric infarction, recent gastrointestinal bleeding, recent stroke, and primary or metastatic central nervous system malignancies.

Endovascular techniques

Before the transjugular approach was attempted, the portal system was studied with indirect portography obtained during the venous phase following iodinated contrast medium injections into the SMA and the splenic artery. Angiography revealed patent superior mesenteric and splenic arteries. Venous phase confirmed complete thrombosis of the SMV and PV.

The transjugular approach was carried out according to the technique previously described[12-14] by using US and fluoroscopic guidance of the portal vein puncture. Following infiltration of local anesthesia, a Rosch-Uchida

Table 1 Summary of clinical data

No. of patients	Age (yr)/sex	Symptoms	Etiologies of splenectomy	Platelet count at admission ($\times 10^3$/mm^3)	Onset of symptoms postoperative day
1	40/M	Fever, abdominal pain, distension, and nausea	Cirrhosis, portal hypertension, and variceal bleeding	540	16
2	43/M	Epigastric pain, diarrhea, fever, and nausea	Cirrhosis, portal hypertension, variceal bleeding, hypersplenism	280	14
3	38/F	Abdominal pain, nausea, distension, and vomiting	Portal hypertension, variceal bleeding	660	11
4	42/M	Abdominal pain, diarrhea, and nausea	Cirrhosis, portal hypertension, variceal bleeding, hypersplenism	360	19
5	32/M	Abdominal pain, distension, nausea, heme-positive stools	Cirrhosis, portal hypertension, variceal bleeding, hypersplenism	240	35
6	52/M	Abdominal pain, nausea, and diarrhea	Portal hypertension, hypertension, variceal bleeding, hypersplenism	340	28

set (Cook, Bloomington, IN, USA) was used to gain access to the portal vein branch. Once the catheter was placed inside a portal branch, the thrombus could be traversed with the aid of a 4 Fr Cobra catheter (Cordis, the Netherlands) and a hydrophilic guidewire (Terumo, Japan). After reaching distal branches of the SMV, the Cobra catheter was exchanged for an 8 mm diameter angioplasty balloon catheter (Boston Scientific, MA, USA), to open up a channel, and then a 10-Fr Rosch-Uchida sheath (Cook) was put into the portal trunk. A bolus of 3000 IU of heparin was injected *via* a peripheral venous catheter.

Through the 10-Fr sheath, an angled 8-Fr guiding catheter (Cordis) was used to aspirate as much of the thrombus as possible from the SMV and PV with a Luer-Lok 60-mL syringe. The aspiration procedure was performed from distal to proximal clots in 8-12 cycles (10 ± 2). Simultaneously, a 5-Fr pigtail catheter (Cordis) was used to fragment the thrombus with "spinning technique"[16] and an injection of urokinase (TIANJIN Biochemical Pharmaceutical Co., LTD, China) 200 000-300 000 IU using a hand-pulse spray technique.

Following the mechanical aspiration procedure, a 4-Fr multiple side-hole catheter (Angiodynamics, Queensbury, NY, USA) was placed with the tip in the SMV, and then continuous thrombolytic therapy was started with urokinase 50 000 IU/h. Heparin infusion was given simultaneously *via* a peripheral venous catheter, at a dose of 1000 IU/h. The adequacy of anticoagulation was adjusted to maintain the activated partial thromboplastin time between 2.0 and 2.5 times the control, during treatment and throughout hospitalization. During the prolonged infusion of thrombolytics, patients had PV-SMV venographic follow-up *via* the infusion catheter every 24 h. Color Doppler ultrasound scan (CDUS) assessment of PV and SMV patency was performed at 24, 48, and 72 h, 1 wk following the procedure, and at discharge.

Termination of the infusion of thrombolytics was based on clinical and radiographic findings. The catheter infusion of thrombolytics was discontinued after the patients' symptoms (i.e. abdominal pain, distention, and diarrhea) had improved sufficiently that they were able to begin oral intake, and the repeated venography demonstrated good flow from the SMV into the portal vein, and no relapse of the thrombosis. The patients were then placed on chronic anticoagulation with warfarin adjusted to maintain an International Normalized Ratio of 2-3 after discharge.

Follow-up US was performed at patient discharge, every 2-3 mo in the first year, and every 4-6 mo in the second year. Follow-up CT was carried out at patient discharge, and then every 3 mo for 1 year and then every 6-12 mo thereafter.

Technical success was defined as successful catheterization of the portal vein, removal of the majority clots, and restoration of flow in the trunk of PV and SMV. Clinical success was defined as relief of symptoms and bowel resection was not required after the procedure. Minor complications were defined as no therapy or normal therapy without consequence.

RESULTS

Technical success

Technical success was achieved in all 6 patients. No complications, such as hemorrhage or contrast extravasation were observed during the procedures. Direct venography of PV-SMV after access to the portal vein confirmed extensive thrombosis in the PV and SMV with poorly formed collateral drainage (Figures 1 and 2).

Using pigtail catheter fragmentation, local urokinase injection, and manual aspiration thrombectomy of the PV-SMV thrombosis resulted in removal of clots $\geqslant$ 60% (60%-80%) in all 6 patients (Figures 3 and 4). Restoration of partial flow in the main PV and SMV was documented on immediate follow-up direct portal venography.

After mechanical thrombolysis, continuous thrombolytic therapy *via* the indwelling infusion catheter in the SMV was performed for three to six days (4.5 ± 1.5 d). The mean total dose of urokinase *via* the catheter infusion was 5.8 million IU (range, 3.6-7.2 million). At completion of indwelling catheter infusion of thrombolytics a near complete lysis of clots (removal of clots greater than 90%) was observed in 4 patients and a partial lysis with a degree of residual thrombus less than 20% was observed in 2 patients, which were confirmed by repeated venography *via* the infusion catheter at the SMV.

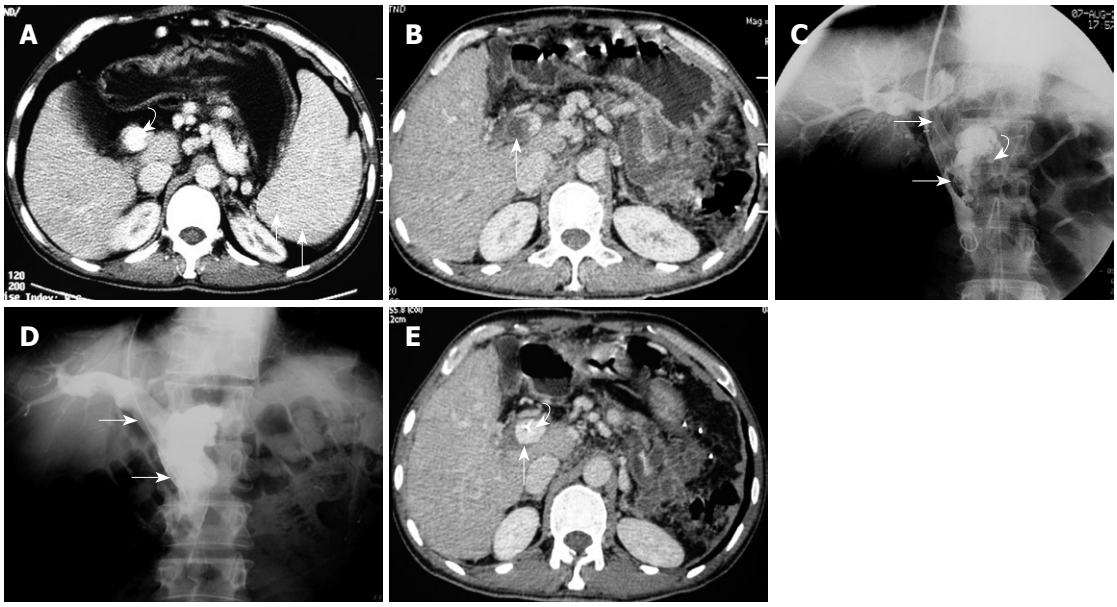

Figure 1 Case 1: A 40-year-old man with low-grade fever, abdominal pain, distension, and nausea for 12 d. He had undergone splenectomy 4 wk previously. A: Selected axial image from before open splenectomy contrast-enhanced CT shows splenomegaly (arrows) and patent portal vein (curved arrow); B: Selected axial image from admission contrast-enhanced CT, on postoperative day 28, shows massive thrombus within the PV (arrow); C: Pre-treatment direct venography *via* transjugular approach access to the portal vein shows massive thrombosis of the PV extending into the SMV (arrows). Note the stump of the splenic vein (curved arrow); D: Follow-up direct portal venography *via* the infusion catheter, obtained 5 d after the catheter infusion of thrombolytics, shows patency of the main PV-SMV (arrows); E: CT image at the same level as in Figure 1B obtained 5 d after the interventional procedure, before the infusion catheter removal, shows the wide patent main PV (arrow). Note the infusion catheter within the PV (curved arrow).

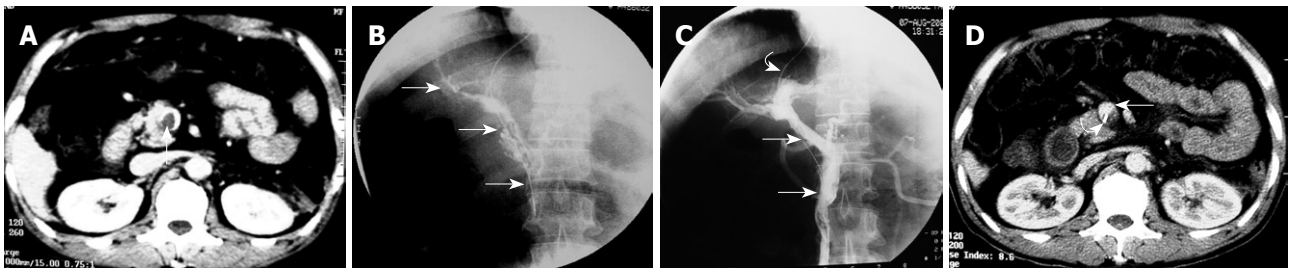

Figure 2 Case 2: A 43-year-old man with epigastric pain, diarrhea, fever, and vomiting for 1 wk. He had undergone splenectomy 3 wk previously. A: Selected axial image from admission contrast-enhanced CT, on postoperative day 21, shows thrombus (arrow) within the SMV; B: Pre-treatment direct venography *via* transjugular approach access to the portal vein shows extensive thrombosis of the SMV extending into the main PV and intrahepatic branches (arrows); C: Follow-up direct portal venography *via* the infusion catheter (curved arrow), obtained 6 d after the catheter infusion of thrombolytics, shows patent PV and SMV with only minimal residual wall thrombus (arrows); D: CT image at the same level as in Figure 2A obtained 6 d after the catheter infusion of thrombolytics, before the infusion catheter removal, shows the wide patent SMV (arrow) with only minimal residual wall thrombus. Note the infusion catheter within the SMV (curved arrow).

Clinical improvement

Sufficient clinical improvement was seen in all 6 patients after 12-24 h of the mechanical thrombolysis procedure, characterized by a progressive reduction in abdominal pain, nausea, diarrhea, and distention. The patients continued to improve clinically during thrombolysis *via* the indwelling infusion catheter in the SMV. Oral intake was started at 3-5 d (4 ± 1.0 d) after abdominal pain, nausea, distention, and diarrhea was completely resolved. No patient required bowel resection after the procedures. No thrombotic, hemorrhagic, or infectious complications were noted during hospitalization. All 6 patients were discharged within 6-14 d (8 ± 2.5 d) of admission.

Contrast-enhanced CT was obtained in all 6 patients before discharge. The images demonstrated almost complete disappearance of PV-SMV thrombosis in 4 patients, and partial recanalization of the PV and SMV with residual thrombus (less than 20% compared to pre-treatment) in 2 patients. Three patients had abnormal AST and ALT values at admission, which returned to normal at discharge.

Follow-up

The mean duration of follow-up after hospital discharge was 40 ± 16.5 mo (range, 15-62 mo). All 6 patients are alive at writing, and no recurrent episodes of PV and SMV thrombosis developed during the follow-up period. Chronic anticoagulation with oral warfarin was initiated in all 6 patients at least 6 mo (6-12 mo) after hospital discharge. During the 15-62 mo follow-up period, US and contrast-enhanced CT confirmed the patency of the PV-SMV, without cavernous transformation of the PV or extrahepatic collaterals.

The platelet count returned to the normal range in

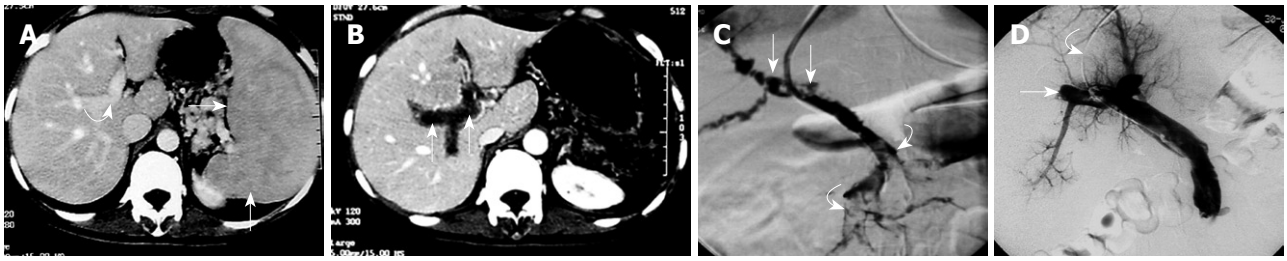

Figure 3 Case 3: A 38-year-old woman with abdominal pain, nausea, distension, and vomiting for 3 d. She had undergone splenectomy 2 wk previously. A: Selected axial image from before open splenectomy contrast-enhanced CT shows splenomegaly (arrows) and patent portal vein (curved arrow); B: Selected axial image from admission contrast-enhanced CT, on postoperative day 14, shows extensive thrombus within the PV (arrows); C: Pre-treatment direct venography *via* transjugular approach access to the portal vein showing extensive PV (arrows) and SMV (curved arrows) thrombosis, without collateral drainage; D: Follow-up direct portal venography *via* the infusion catheter (curved arrow), obtained 5 d after the catheter infusion of thrombolytics, shows patent PV and SMV with residual thrombosis within the intrahepatic portal venous branches (arrow).

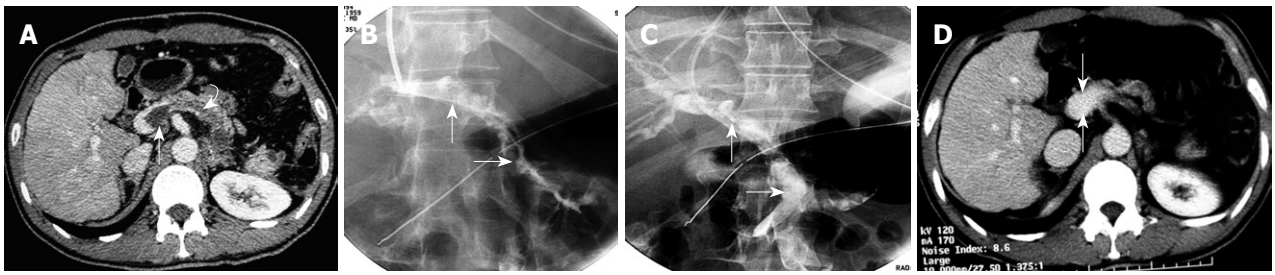

Figure 4 Case 4: A 42-year-old man with abdominal pain, diarrhea, and nausea for 2 wk. He had undergone splenectomy 4 wk previously. A: Selected axial image from admission contrast-enhanced CT demonstrates massive thrombus within the proximal of SMV (arrow) and the stump of the splenic vein (curved arrow); B: Pre-treatment direct venography *via* transjugular approach access to the portal vein showing extensive PV-SMV thrombosis (arrows), without collateral drainage; C: Immediate follow-up direct portal venography after catheter fragmentation and aspiration of the PV-SMV thrombosis, shows 75% reduction of thrombosis in the SMV and PV (arrow). The catheter infusion of thrombolytics was continued for 4 d and further improvement was confirmed by CT follow-up; D: Contrast-enhanced CT image at the same level as in Figure 4A obtained 5 d after completion of the interventional procedure shows a wide patent SMV (arrows).

four patients. Long term oral aspirin was given at a dose of 100 mg/d to 2 patients because their platelet count was > $300 \times 10^3/mm^3$ ($380 \times 10^3/mm^3$, $460 \times 10^3/mm^3$, respectively, at the last examination).

DISCUSSION

Although the exact mechanisms of PV-SMV thrombosis formation after splenectomy remain unclear, altered platelet function as well as transient thrombocytosis after splenectomy, a decrease in portal blood flow and pressure, and stasis of blood in the stump of the splenic vein appear to predispose to PV-SMV thrombosis[6,18]. Ikeda *et al*[4] reported that patients with PV-SMV thrombosis after splenectomy had a significantly heavier splenic weight than those without PV-SMV thrombosis, suggesting that a large splenic mass is a possible risk factor for post-splenectomy PV-SMV thrombosis. Stamou *et al*[7] reported that a platelet count of more than $650 \times 10^3/mm^3$ and greater spleen weight (> 650 g) was associated with the development of portal system thrombosis. In our cases, marked splenomegaly was present in all 6 patients. In addition, a significant increase in platelet count was found in 4 patients at admission. However, we can not draw any conclusions due to our very small group of patients and lack of a control group.

Medical and surgical options are of limited value in extensive PV-SMV thrombosis[1,2]. In symptomatic PV-SMV thrombosis patients, treatment depends on the presence or absence of clinical and CT peritoneal signs. An emergency laparotomy with resection of necrotic bowel is necessary in the former condition and anticoagulant and/or thrombolytic therapy in the latter condition. The advantage of surgical embolectomy is that it allows for direct inspection of the bowel at the time of embolectomy and resection of necrotic bowel, if necessary. However, it is often difficult to remove all the thrombus from the small branches of the mesenteric veins. Often, adjuvant thrombolytic therapy is necessary and can be associated with a high risk of bleeding in the postoperative patient[19,20]. Systemic anticoagulant and/or thrombolytic therapy is of limited value in extensive PV-SMV thrombosis, as it has low efficacy and is time consuming[2].

In patients with acute or subacute symptomatic PV-SMV thrombosis, endovascular interventional treatment has been reported with encouraging initial results[10-17]. With this approach, PV-SMV thrombosis can be managed by pharmacologic thrombolysis and/or mechanical thrombectomy. For pharmacologic thrombolysis, possible routes of treatment include indirect intra-arterial infusion of thrombolytic agents *via* the SMA[10,11] and direct access to the portal vein, by the transjugular[12-14] or transhepatic routes[15-17]. Mechanical thrombectomy techniques include balloon angioplasty, thrombectomy devices, aspiration thrombectomy, stent placement, and TIPS creation[14,15].

Indirect thrombolytic therapy *via* the SMA is less technically demanding and has been described for its potential benefits in infusing thrombolytic agents into small mesenteric venous branches[10,11]. However, this approach does not allow direct infusion into the thrombus, may result in lytic agents diverting through patent branches and collaterals, and possible prolongation of the total infusion time *via* the SMA[12], which may result in an increased risk of bleeding. Direct access to the portal vein by a transjugular or transhepatic route directly targets the PV-SMV thrombosis, leading to fast removal of the thrombus and flow improvement, and an improvement in the patient's symptoms[14,15].

Percutaneous transhepatic access is technically relatively easy and allows the maneuver of mechanical devices compared with transjugular intrahepatic access. Usually, this approach is suitable for the removal of larger clots within the trunk of the PV and SMV. The drawbacks of the percutaneous transhepatic route include the development of intraperitoneal or subcapsular hepatic hemorrhage[16,21]. This is likely to occur given that the transhepatic route for mechanical thrombectomy of splanchnic venous thrombosis requires traversing the hepatic capsule and is followed by thrombolysis and possibly systemic anticoagulation.

Our 6 patients with acute extensive PV-SMV thrombosis after splenectomy treated with the transjugular intrahepatic approach demonstrates the feasibility of this route to the management of this challenging illness. The transjugular approach access to the portal vein is generally performed with the creation of a transjugular intrahepatic portosystemic shunt; this approach is usually indicated for patients with cirrhosis with portal hypertension caused by portal vein thrombosis[13,14]. Compared to the percutaneous transhepatic approach, the transjugular intrahepatic approach does not require traversing the hepatic capsule, and thus would eliminate the risk of subcapsular hemorrhage[12,14]. Furthermore, the transjugular intrahepatic approach is safer in patients with anticoagulation. In addition, based on our experience with the TIPS procedure, we opted for this approach to treat an extensive acute PV-SMV thrombosis. Although we did not observe any complications in our 6 patients with the transjugular approach, significant intra-abdominal bleeding is a potential serious complication[22].

Mechanical thrombectomy devices and aspiration thrombectomy are feasible and effective in the re-establishment of portal and mesenteric circulation in patients with acute extensive thrombosis[12,21]. Good results have been obtained with thrombectomy devices such as the Arrow-Trerotola, Oasis, Amplatz thrombectomy, and AngioJet, although the clinical experience with these thrombectomy devices in PV-SMV thrombosis is limited[12,23,24]. We did not use mechanical thrombectomy devices in our small series because these devices were unavailable at that time in our angiographic laboratory. Aspiration of the fresh thrombus with a large lumen catheter has also been reported[14,25]. The advantages of this technique are that a large-lumen catheter is generally available in standard angiography laboratories, is of low cost compared with the various thrombectomy devices, and is an easy device which can be as effective as the various thrombectomy devices in removing thrombus from a vessel[16,25].

In patients with acute extensive thrombosis of the PV-SMV, mechanical thrombectomy could initially be used to debulk the thrombus, and pharmacologic thrombolysis would probably still be necessary in most cases to treat residual thrombosis and to treat thrombus in the small and peripheral veins[16,23]. The combination of aspiration and local pharmacological thrombolysis *via* a direct access to the portal system is more effective and significantly decreases the treatment time in patients with extensive PV-SMV thrombosis compared to indirect and direct thrombolysis infusion alone[12,23,24]. In our 6 cases, aspiration thrombectomy associated with indwelling catheter infusion of thrombolytics into the SMV was effective, resulted in a rapid improvement in symptoms, recanalization of the SMV, resolution of symptoms, and resumption of oral nutrition.

Combining thrombolytic infusion with anticoagulation would appear to increase the risk of bleeding and hemorrhage[26,27]. A study by Ouriel *et al*[26] described the complication rates for patients with lower-extremity arterial or venous occlusions treated with local urokinase or rt-PA. Overall, 15% required transfusion and 1.2% developed intracranial hemorrhage, which was fatal in 8 of 9 cases. In our series, no bleeding complications occurred. This may have resulted from the relatively low dose infusion of urokinase *via* the catheter in the SMV, no simultaneous peripheral venous infusion of urokinase, and careful monitoring of the coagulation status during treatment. As for thrombolytic agents, we prefer urokinase to rt-PA (recombinant tissue plasminogen activator), because it is similarly active on thrombus dissolution but appears to be safer, being associated with a lower incidence of hemorrhagic complications[26]. In addition, urokinase is generally available in our institution, and is less costly than rt-PA.

Interventional endovascular thrombectomy and direct thrombolysis can offer a non-surgical alternative for the treatment of extensive PV-SMV thrombosis[10-17]. However, this can only be performed in a select group of patients who do not present with bowel ischemia and infarction, or who are not at risk for bleeding, and who have persistent symptoms or worsening of symptoms despite anticoagulation[1,2]. Minimally symptomatic or asymptomatic patients with PV-SMV thrombosis may best be treated with systemic anticoagulation only. Prompt surgical intervention should be undertaken if the patient's condition deteriorates or clinical signs of peritonitis develop during the interventional treatment[2]. In this small group, we chose interventional procedures to treat these patients because systemic anticoagulation was ineffective and because the SMV was involved. The results obtained in our series of patients can be considered satisfactory. All 6 patients showed a patent SMV and PV, without recurrent episodes, during a mean follow-up of 40 mo.

The limitations of this study include the lack of a control group, randomization, and uniformity of

evaluation and treatment. Because of the small sample size, no statistically significant conclusions could be drawn regarding treatment with respect to dosages of thrombolytic agent or heparin, techniques, or underlying risk factors. This is partly related to the low incidence of the illness as well as the natural evolution of therapeutic techniques during the last 10 years.

In summary, the combination of catheter fragmentation of clots, aspiration thrombectomy, and indwelling catheter infusion of thrombolytics *via* transjugular intrahepatic access to the portal system, is a safe and effective therapy for the management of patients with acute extensive PV-SMV thrombosis. Because of the small size of the study and other factors, the ability to generalize the results is limited.

COMMENTS

Background

Thrombosis of the portal vein (PV) and superior mesenteric vein (SMV) is considered a possible cause of death after splenectomy. The reported incidence of PV-SMV thrombosis after elective open splenectomy ranges from 6.3% to 11%. Although many patients with PV-SMV thrombosis may be asymptomatic, the consequences of these thromboses can be severe, including mesenteric ischemia and variceal bleeding, with a mortality rate of 5%-37%. There are no uniform protocols for the effective treatment of PV-SMV thrombosis following splenectomy.

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, new possibilities, such as percutaneous techniques, have been investigated.

Innovations and breakthroughs

The authors report 6 patients with acute extensive PV-SMV thrombosis after splenectomy treated with transjugular approach catheter-directed thrombolysis, which demonstrated the feasibility of this route in the management of this challenging illness. Compared to the percutaneous transhepatic approach, the transjugular approach does not require traversing the hepatic capsule, and thus eliminates the risk of subcapsular hemorrhage. In addition, the transjugular approach is safer in patients with anticoagulation. Secondly, this is the first study to report on the efficacy of the combination of aspiration thrombectomy with an indwelling catheter infusion of thrombolytics into the SMV, which resulted in a rapid improvement in symptoms, recanalization of the SMV, and resolution of symptoms. Finally, the combination of thrombolytic infusion with anticoagulation can be associated with a high risk of bleeding. In the authors' series, no bleeding complications occurred. This may have resulted from the relatively low dose infusion of urokinase *via* the catheter in the SMV, no simultaneous peripheral venous infusion of urokinase, and careful monitoring of the coagulation status during treatment.

Applications

Interventional endovascular thrombectomy and direct thrombolysis can offer a non-surgical alternative for the treatment of extensive 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

Endovascular interventional techniques for the treatment of thrombosis include intra-vascular pharmacologic thrombolysis and mechanical thrombectomy. Catheter-directed intra-vascular thrombolysis can directly target the thrombus, leading to fast removal of the thrombus and flow improvement, and an improvement in symptoms. Interventional mechanical thrombectomy techniques include balloon angioplasty, thrombectomy devices, aspiration thrombectomy, stent placement, and TIPS creation.

Peer review

This study reports 6 patients that were treated with techniques of interventional radiology to restore patency of portal venous thrombosis (extending to the mesenteric vein) that developed after being submitted to surgical splenectomy. The authors report a 100% success rate of the technique without side effects. Although the size of the series is small, the results can be considered satisfactory and is encouraged.

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BRIEF ARTICLES

Comparative identification of Ca^{2+} channel expression in INS-1 and rat pancreatic β cells

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Abstract

AIM: To identify and compare the profile of Ca^{2+} channel subunit expression in INS-1 and rat pancreatic β cells.

METHODS: The rat insulin-secreting INS-1 cell line was cultured in RPMI-1640 with Wistar rats employed as islet donors. Ca^{2+} channel subunit expression in INS-1 and isolated rat β cells were examined by reverse transcription polymerase chain reaction (RT-PCR). Absolute real-time quantitative PCR was performed in a Bio-Rad iQ5 Gradient Real Time PCR system and the data analyzed using an iQ5 system to identify the expression level of the Ca^{2+} channel subunits.

RESULTS: In INS-1 cells, the L-type Ca^{2+} channel 1C subunit had the highest expression level and the TPRM2 subunit had the second highest expression. In rat β cells, the TPRC4β subunit expression was dominant and the expression of the L-type 1C subunit exceeded the 1D subunit expression about two-fold. This result agreed with other studies, confirming the important role of the L-type 1C subunit in insulin-secreting cells, and suggested that non-voltage-operated Ca^{2+} channels may have an important role in biphasic insulin secretion.

CONCLUSION: Twelve major Ca^{2+} channel subunit types were identified in INS-1 and rat β cells and significant differences were observed in the expression of certain subunits between these cells.

Key words: L-type calcium channels; Expression profile; Insulin-secreting cells; Rats; pancreatic β cell; Reverse transcription-polymerase chain reaction

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INTRODUCTION

Recent theories portray type 2 diabetes mellitus (T2DM) as a heterogeneous disorder. In addition to insulin resistance, clinical studies in humans and animals have documented a variety of defects in β cell function[1], and most researchers agree that both insulin secretion impairment and insulin resistance contribute to the fully established disease[2]. Insulin produced by pancreatic islet β cells efficiently regulates glucose homeostasis in humans and other mammals. These cells are electrically excitable, and couple changes in blood glucose concentration to insulin release *via* electrical signals.

Glucose-stimulated insulin secretion is biphasic, with about a 10 min first phase and a several hour second phase[3]. Intracellular Ca^{2+} signals play a pivotal role in β cell function and, as insulin secretion is the most important role of these cells, knowledge of the intricacies of the signals involved in excitation-secretion coupling is important in understanding both normal β cell function and related pathological states. It has been reported that both voltage-dependent and non-voltage-operated Ca^{2+} channels are involved in these processes. The voltage-dependent Ca^{2+} channels include L-type, T-type, N-type and R-type channels[4,5]; the non-voltage-operated Ca^{2+} channels include ryanodine-sensitive Ca^{2+} channels[6-8],

transient receptor potential channels (TRP)[9-11], and inositol 1,4,5-trisphosphate (IP_3)-sensitive channels[12], the latter mobilizing Ca^{2+} from the endoplasmic reticulum. Impaired first-phase insulin secretion is an early feature of T2DM, whereas second-phase insulin secretion deteriorates with progression of the disease. A genetic study has indicated that polymorphisms in R-type channels in humans are associated with T2DM and impaired insulin secretion[13]. Moreover, most Ca^{2+} channels consist of various subunits which participate in different physiologic functions in different species or sub-cloned cell lines[12]. For example, T-type Ca^{2+} channels have little or no expression in rodents, but can be detected in humans[14]. Thus, the above suggests that Ca^{2+} channel subunits may be suitable candidates as pathogenetic factors in diabetes.

Considering the essential functions of Ca^{2+} signals for insulin secretion and the various Ca^{2+} channel subunits in β cells, a systemic identification of Ca^{2+} channel subunit expression in β cells is necessary to further the understanding of their functions in regulating insulin secretion and to find potential new therapy targets for T2DM. The aim of this study was to detect the expression profile of six voltage-dependent Ca^{2+} channel subunits, including L-type (α1C, D, S and 1F subunits), R-type (α1E subunit), and N-type (α1B subunit), and nine non-voltage-operated Ca^{2+} channel subunits including Ryr1, Ryr2, TRPC1, TRPC4α, TRPC4β, TRPM2, IP_3R1, IP_3R2 and IP_3R3, in an INS-1 cell line, and to compare the identified subunits with those detectable in rat primary pancreatic β cells. The INS-1 rat cell line was employed as a model of pancreatic β cells because INS-1 cells show susceptibility to glucotoxicity, similar to β cells.

MATERIALS AND METHODS

Cell culture

INS-1 cells lines were glucose-responsive and a gift from Professor Tao Xu (Institute of Biophysics, Chinese Academy of Sciences, P. R. China) and were cultured in RPMI-1640 medium containing 11.2 mmol/L glucose, 100 mL/L fetal calf serum, 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate[15], 100 000 U/L penicillin, 100 mg/L streptomycin, and 50 mmol/L β-mercapto-ethanol, in a fully humidified atmosphere containing 50 mL CO_2 per liter air at 37℃.

Preparation of rat pancreatic β cells and identification by reverse transcription-polymerase chain reaction (RT-PCR)

Pathogen-free, inbred, male Wistar rats weighing 300-350 g were used as islet donors. The animals were maintained on standard rat chow and acidified water *ad libitum*. For islet retrieval, individuals were sacrificed by cervical dislocation and the pancreas was quickly removed. Pancreatic islets were isolated using a standard collagenase digestion[16,17]. Briefly, islets were separated from exocrine tissue by centrifugation over a discontinuous dextran gradient after digestion with 0.5 g/L collagenase V (Sigma C9263) for 30 min, and further purification by handpicking under a microscope. Islets were collected and washed twice in phosphate-buffered saline and dispersed as single cells by mechanical shaking in 4 mL of Hank's solution (Ca^{2+} and Mg^{2+} free) before filtering the cell mixture over a 35 μm pore size filter (BD Falcon) and diluting to 200 mL. This preparation was divided into 20 mL per tube, and cDNA was synthesized from the total isolated RNA, as described below.

To distinguish glucagon-producing-cells from insulin-producing-cells, specific primer pairs were designed from the insulin and glucagon genes in GenBank. The sequences were: insulin-F, AAACAGCACCTTTGTGG TTCTCA; insulin-R, GTGCCACTTGTGGGTCCTCC; glucagon-F, TCGTGGCTG GATTGTTTG; and glucagon-R, TGGCGTTTGTCTTCGTTTAT. The PCR procedure was: the insulin gene at 95℃ for 30 s, 59℃ for 30 s, 72℃ for 30 s, and 35 cycles; and the glucagon gene at 95℃ for 30 s, 53℃ for 30 s, 72℃ for 30 s, and 35 cycles.

RT-PCR and absolute real-time quantitative PCR analysis

Total RNA was extracted from INS-1 cells and rat pancreatic β cells using the RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. One microliter aliquot of the RNA was incubated with Oligo (dT) 18 at 70℃ for 5 min, and then put on ice. RNase inhibitor (1 μL), 100 μmol/L dNTPs, 0.01 mol/L dithiothreitol, and 200 U of M-MLV reverse transcriptase (Promega, USA) were added to the mixture (20 μL final volume), incubated at 42℃ for 50 min, and then incubated at 85℃ for 5 min. Table 1 summarizes the primer pairs used for the amplification of Ca^{2+} channel subunits, PCR was performed in a standard 50 μL reaction volume, and the resulting products were visualized by Golden view after 3% agarose gel electrophoresis in TAE buffer (40 mmol/L Tris-acetate, 2 mmol/L EDTA, pH 8.5).

The expression levels of the subunits were quantified by absolute real-time quantitative PCR with the Bio-Rad iQ5 Gradient Real Time PCR system and all reactions performed in a 25 μL reaction volume containing 12.5 μL of 2xSYBR Green Master mix (Bio-Rad, USA), 1 μL of each primer pair (10 μmol/L), and 1 μL of cDNA templates. The products of PCR were cloned into the pGEM-T easy vector using the pGEM-T easy cloning kit (Promega, USA). Five microliters LB-broth cultures containing single colonies were grown up overnight at 37℃ with shaking at 200 r/min and the resulting plasmids were purified. Plasmid DNA was solubilized in 50 μL TE buffer and the sequences of the cloned products confirmed by DNA sequencing. Purified plasmid clones were quantified by Eppendorf Biophotometer and the copy number calculated as follows: 6.02×10^{23} (copies/mol) × DNA amount (g)/[DNA length (bp) × 660 (g/mol per becquerel)].

Based on the copy number and concentration of the plasmid DNA, the precise number of molecules added to subsequent real-time PCR runs was calculated, thus

Table 1 Sequences of primer pairs of Ca^{2+} channel subunits and RT-PCR

Primer	Sequence	Temperature (℃)
L-α1C (5')	5'-GTCCATAGTGGGTCGTCT-3'	50
L-α1C (3')	5'-TGGTTTGTTCTTGCTTTC-3'	
L-α1D (5')	5'-CAGGGATGCTGTGGAAGT-3'	51
L-α1D (3')	5'-TGGGCTGAGAACCTAGACG-3'	
L-α-S (5')	5'-ATGCCAGAGGATGACAACAAC-3'	55
L-α-S (3')	5'-CACCCAgAAAgACAATGATgAA-3'	
L-α1F (5')	5'-GACGGCAACTTGGCTTCT-3'	53
L-α1F (3')	5'-GCTGGCATGACTGCTGGT-3'	
N-α1β(5')	5'-GCTCGCTCTTCGTCTTCA-3'	52
N-α1β (3')	5'-AGGTTCCGTGTCATCCAGT-3'	
R-α1E (5')	5'-ATGTCCCTGAAGATGTATGG-3'	50
R-α1E (3')	5'-AACGACCTCAAAGATGCTG-3'	
Ryr1 (5')	5'-GGGCGGAGAATGAGAAAGA-3'	55
Ryr1 (3')	5'-CAGGGTCGTCACGGTTGT-3'	
Ryr2 (5')	5'-TGAGTATGCCCATTGAGT-3'	48
Ryr2 (3')	5'-CTTTGCTTTAGGCGTGAG-3'	
TRPC1 (5')	5'-GTCAGACATTAAGAGGCTGTG-3'	50
TRPC1 (3')	5'-AAGTTGCCAAGTAAAGGGA-3'	
TRPC4α (5')	5'-AATGGTTCTGCCCTGGTG-3'	50
TRPC4α (3')	5'-GAAGATTTGGTTTGCGTTT-3'	
TRPC4β (5')	5'-GCAGCATTCCTGGTCTCA-3'	51
TRPC4β (3')	5'-GGGCGTGTTTCTCCTTTG-3'	
TRPM2 (5')	5'-GTCATCACCATCGGCATAGC-3'	56
TRPM2 (3')	5'-TGTCCAGGCAGGTCAGGTT-3'	
IP_3R1 (5')	5'-CAACCGTTACTATGGAAACATC-3'	54
IP_3R1 (3')	5'-TCAGCCAGGCTCATCTCAC-3'	
IP_3R2 (5')	5'-CGATGCCAGGATACGATGT-3'	54
IP_3R2 (3')	5'-CACCCTTGAAGTACCGATT-3'	
IP_3R3 (5')	5'-AGGAGCTGGTGGACGTGAT-3'	55
IP_3R3 (3')	5'-TGCTTGTTGTGCCTGGAAA-3'	

Table 2 Expression profile and clone numbers of Ca^{2+} channel subunits in INS-1 and rat primary β cells (mean ± SE)

Ca^{2+} channel subunits	Length of PCR production (bp)	INS-1	β cells
L-α1C	147	$1.767 \times 10^7 \pm 1.763 \times 10^6$	$3.836 \times 10^6 \pm 1.087 \times 10^6$
L-α1D	197	$1.046 \times 10^6 \pm 2.074 \times 10^4$	$1.913 \times 10^6 \pm 1.329 \times 10^5$
L-α-S[1]	181	-	-
L-α1F[1]	144	-	-
N-α1β	188	$1.078 \times 10^4 \pm 6.477 \times 10^2$	$6.832 \times 10^3 \pm 3.220 \times 10^2$
R-α1E	102	$6.614 \times 10^3 \pm 1.477 \times 10^2$	$2.84 \times 10^3 \pm 1.943 \times 10^2$
Ryr1[1]	159	-	-
Ryr2	250	$1.367 \times 10^4 \pm 7.213 \times 10$	$6.520 \times 10^5 \pm 4.864 \times 10^4$
TRPC1	101	$5.631 \times 10^4 \pm 1.904 \times 10^3$	$3.260 \times 10^6 \pm 2.531 \times 10^5$
TRPC4α	196	$8.033 \times 10^4 \pm 5.405 \times 10^3$	$2.073 \times 10^6 \pm 2.207 \times 10^5$
TRPC4β	165	$2.226 \times 10^5 \pm 7.672 \times 10^4$	$6.666 \times 10^6 \pm 1.595 \times 10^6$
TRPM2	130	$1.028 \times 10^6 \pm 9.696 \times 10^4$	$1.538 \times 10^6 \pm 2.476 \times 10^5$
IP_3R1	159	$1.189 \times 10^5 \pm 1.650 \times 10^4$	$1.922 \times 10^6 \pm 7.584 \times 10^5$
IP_3R2	191	$3.339 \times 10^4 \pm 7.945 \times 10^3$	$8.293 \times 10^4 \pm 7.041 \times 10^3$
IP_3R3	130	$5.613 \times 10^5 \pm 1.009 \times 10^5$	$7.863 \times 10^5 \pm 3.894 \times 10^3$

[1]These three subunits were not identified in INS-1 and rat β cells.

providing a standard for specific cDNA quantification. All samples were prepared as 1:10, 1:100, and 1:1000 dilutions and each reaction at different dilutions performed in triplicate. The standard curve and data analysis were produced using Bio-Rad iQ5 software.

RESULTS

Preparation of rat pancreatic β cells and identification by RT-PCR

An average of 300-500 islets were produced from each rat, with about 1000 cells per islet and composed of 70% β cells. In the first 10 cDNA templates analyzed, six templates only expressed the insulin gene, three expressed the glucagon gene, and the last one expressed neither. The cDNA whose glucagon gene was positive was considered to be from contamination by alpha islet cells and could be ignored and, thus, the others were chosen as templates for analysis of β cell Ca^{2+} channel subunit expression.

Expression of Ca^{2+} channels subunits in INS-1 and rat pancreatic β cells

RT-PCR was performed to identify the expression of Ca^{2+} channel subunits in INS-1 and rat pancreatic β cells. Of the 15 subunit types, 12 types were amplified from INS-1 and rat pancreatic β cells and their identities confirmed (Figure 1A). Three types, not identified in either INS-1 or rat pancreatic β cells, were the L-type α1F, S and Ryr1 subunits. Under the same reaction conditions, these three were identified in cDNA from heart and skeletal muscle because these sources

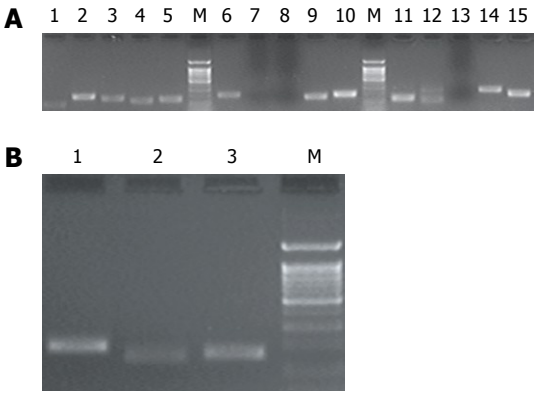

Figure 1 The Ca^{2+} channel subunits expression profile in INS-1 and rat β cells. A: 12 of 15 Ca^{2+} channel subunits in rat pancreatic β cells. 1-5: Separately represent subunits TRPC1, TRPC4α, TRPC4β, TRPM2, and L-α1C; 6-10: Separately represent subunits L-α1D, L-α-S, L-α1F, IP_3R1, and IP_3R2; 11-15: IP_3R3, R-α1E, Ryr1, Ryr2, and N-α1B; M: 100 bp ladder marker, (bottom band, 100 bp; the expression profile of INS-1 is the same as for rat β cells, not shown); B: L-α-S, L-α1F, and Ryr1 expressed in rat heart and skeletal muscle. 1-3: L-α-S (cDNA of skeletal muscle), L-α1F (heart), and Ryr1 (cDNA of skeletal muscle); M: 100 bp ladder marker; bottom band of M, 100 bp.

are composed of multiple tissues, including muscles, blood vessels, and nerve fibers (Figure 1B). It was found that 12 subunit types were expressed in INS-1 and rat pancreatic β cells (Table 2): L-type (α1C, α1D subunits), R-type (α1E subunit), and N-type (α1β subunit) preferentially in INS-1 cells; Ryr2, TRPC1, TRPC4α, TRPC4β, TRPM2, IP_3R1, IP_3R2, and IP_3R3 preferentially in β cells.

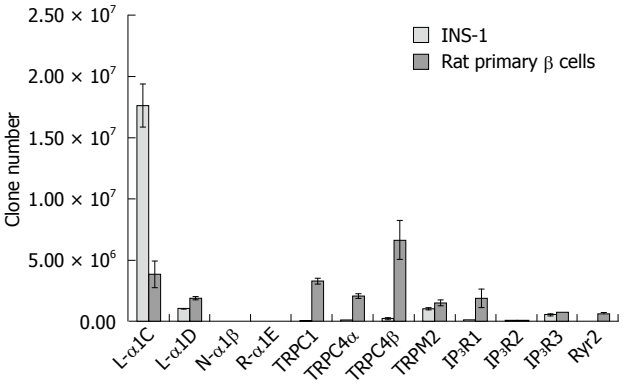

Figure 2 Expression level of 12 Ca^{2+} channel subunits in INS-1 and rat primary β cells.

mRNA level of Ca^{2+} channel subunits in INS-1 and rat pancreatic β cells

Melt curve analysis of all subunits revealed that there was a single peak at the expected melting temperature for PCR applications. The absolute real-time quantitative PCR analyses showed (Table 2, Figure 2) that, in INS-1, L-type α1C subunits were dominant and were expressed significantly more than other subunits. The R-α1E subunit was expressed at a very low level as in the β cells. In the latter cells, the expression levels of subunits were relatively similar, but the expression of Ryr2, TRPC1, TRPC4α, TRPC4β, TRPM2, and IP_3R1 were significantly higher than in INS-1.

DISCUSSION

As important regulatory factors in insulin secretion, Ca^{2+} channels have potential as targets for developing new T2DM therapies. Considering the variety of Ca^{2+} channel subunits present in different cell clones and species, the aim of this study was to perform a systematic identification of Ca^{2+} channel subunits in INS-1 and rat pancreatic β cells. Collectively, 12 of 15 subunit types were found to be expressed in INS-1 and primary rat islet β cells. The L-type α1C subunit expression exceeded that of α1D 10-fold in INS-1 cells, but only 2-fold in the β cells. These results were similar to recent research in INS-1 832/12 cells which confirmed that α1C subunit expression exceeded that of the α1D subunit by two-fold and that the α1C subunit had a critical role in insulin secretion[18]. The L-type α1C subunit performs a special function in the first phase of insulin secretion and glucose tolerance. In mice, α1C subunit deficiency decreased the whole-cell Ca^{2+} current by about 45% and abolished the first phase of insulin secretion, resulting in glucose intolerance[19]. In the present study, L-type α1C subunits were dominant among all subunit expressions, which was in accordance with its important biological function in both INS-1 and rat β cells. The L-type α1D subunit's roles in insulin secretion or proliferation were not confirmed until recently[18,20-22].

The expression of the non-voltage-operated Ca^{2+} channel subunits Ryr2, TRPC1, TRPC4α, TRPC4β, and IP_3R1 in rat β cells exceeded those in INS-1 by 10-fold and TRPM2 expression was not significantly different between the β cells and INS-1. These results suggested that non-voltage-operated Ca^{2+} channels may have a greater role in regulating insulin secretion in comparison with voltage-dependent Ca^{2+} channels. The expression level of the TRPC4β subunit was dominant in the β cells and, as a member of the TRPC subfamily, TRPC4 shows four protein motifs (M1-M4) characteristic of the TRPC sub-family[23]. Specifically, TRPC4β lacks 84 amino acids in the C-terminus, which corresponds to putative binding sites for calmodulin and IP_3 receptors in TRPC4α. The ionic channels formed by TRPC4 appear to be Ca^{2+}-permeable, but there is a considerable discrepancy in the degree of Ca^{2+} selectivity. Studies with mice lacking TRPC4 suggest an important role for TRPC4 in supporting Ca^{2+} entry[24]. The defect in Ca^{2+} entry in TRPC4-/- mice appears to be associated with a reduction in arterial vasorelaxation, vascular permeability in the lung, and neurotransmitter release from thalamic dendrites[25]. Though the expression levels of subunits observed here were not absolutely coupled with their functions, the present results suggested that TRPC4β may be performing some functions in insulin biphasic secretion and that further clarification is needed.

The insulin-secreting INS-1 cell line was established by dispersion of a radiation-induced insulinoma from NEDH rats in 1992[15]. INS-1 cells can respond to glucose and are generally considered to be a β cell model, but an important drawback to this cell line is its polyclonal nature reflected by the presence of glucose-responsive and glucose-unresponsive subpopulations[26]. As was demonstrated here, there was a significant difference in the levels of expression of Ca^{2+} channel subunits between INS-1 and β cells, which probably reflected differences in the intracellular metabolism and/or secretory pathways. Taken together, these INS-1 cells may not have represented an exclusively insulin-producing β cell line.

The present study systematically identified the expression profile of Ca^{2+} channel subunits in INS-1 cells and rat pancreatic β cells and quantitatively characterized them by direct comparison. These results will be helpful in advancing the understanding of Ca^{2+} channel subunits and their roles related to insulin secretion.

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COMMENTS

Background

The proportion of people with type 2 diabetes has increased throughout the world. It is now recognized that abnormal insulin secretion precedes the onset of type two diabetes. Ca^{2+} channels have important roles in the progress of insulin secretion by β cells.

Research frontiers

Ca^{2+} channels are composed of several subunits and mainly control Ca^{2+} influx through different mechanisms because of different patterns of subunit composition. In islet β cells, the expression profile of these subunits has not

been systematically investigated. In this study, the authors systematically identified the expression of Ca^{2+} channels in primary β cells and INS-1, an insulin-secreting rat cell line.

Innovations and breakthroughs

This is the first study to report the expression profile of Ca^{2+} channel subunits in primary rat pancreatic β cells and INS-1. Furthermore, the real time PCR data suggested that the INS-1 cell line was not an ideal β cell bioelectrical model.

Applications

By understanding which types of Ca^{2+} channels are expressed in rat β and INS-1 cells, this study may have advanced the understanding of the possible functions of various Ca^{2+} channels in insulin secretion and provided clues to the physiopathology of diabetes.

Terminology

Ca^{2+} channels provide pores for the passive diffusion of ions across biological membranes, in particular Ca^{2+}. β cells are the unique cells of insulin production, and the INS-1 is an insulin-secreting cell line established by dispersion of a radiation-induced insulinoma from NEDH rats in 1992.

Peer review

This is an interesting study, the authors identified the expression profile of Ca^{2+} channel subunits in the INS-1 cell line and rat pancreatic β cells, by reverse transcription polymerase chain reaction.

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Measuring the space between vagina and rectum as it relates to rectocele

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Abstract

AIM: To measure the normal space between the posterior wall of the vagina and the anterior wall of the respectively rectum using computed tomography (CT) and reveal its were relationship to rectocele.

METHODS: A total of twenty female volunteers without rectocele were examined by CT scan. We performed a middle level continuous horizontal pelvic scan from the upper part to the lower part and collected the measurement data to analyze the results using *t*-test.

RESULTS: Twenty volunteers were enrolled in the study. The space between the posterior wall of the vagina and the anterior wall of the rectum was measured at three levels (upper 1/3, middle, lower 1/3 level of vagina). The results showed that the space from the posterior wall of the vagina to the anterior wall of the rectum at the upper 1/3 level and the middle level was 3.896 ± 0.3617 mm and 4.6575 ± 0.3052 mm, respectively. When the two groups of data were compared, we found the space at the upper 1/3 level was shorter than at the middle level ($P < 0.01$). Moreover, at the lower 1/3 level the space measured was 10.058 ± 0.4534 mm. The results revealed that the space at the lower 1/3 level was longer than that at the middle level ($P < 0.01$).

CONCLUSION: These measurement data may be helpful in assessing rectocele clinical diagnosis and functional outcomes of rectocele repair.

Key words: Measurement; Space; Rectocele; Computed tomography

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INTRODUCTION

Rectocele (herniation of the anterior rectal wall into the posterior wall of vagina) is a common problem in women. Nichols and Genadry[1] and Pucciani[2] divided rectocele into those with chronic evacuation difficulty and normal genital position (type 1, distension rectocele) and those associated with genital organ prolapse (type 2, displacement rectocele). It has been suggested that these two types have different anatomical, clinical, and therapeutic methods. In the past few decades, several techniques have been proposed for treating rectocele. In some mild cases, conservative management succeeded. If conservative management failed to relieve symptoms, surgical treatment was advocated. Endorectal rectocele repair has been performed by colorectal surgeons[3-5]. However, after endorectal repair difficult evacuation has been reported[6,7]. Transvaginal rectocele repair has been performed mainly by gynecologists using posterior colporrhaphy[8,9]. These methods included plication of the levator muscles, strengthening of the rectovaginal septum and closure of the specific defect of the rectovaginal facia. Transvaginal repair has been criticized because of sexual discomfort. The aim of the present study was to measure the normal spaces between rectum and vagina using computed tomography (CT). To our knowledge, data on the normal space between rectum and vagina was lacking. This information should prove beneficial not only to rectocele repair but also to the correction of genital organ prolapse.

MATERIALS AND METHODS

Twenty female volunteers without rectocele were examined by CT (SIEMENS SOMATOM spirit, JAPAN) scan in Tianjin HongQiao hospital. Their mean age were 42.5 years (range 32-48 years). They were placed in the supine position, and received continuously horizontal pelvic scan from the upper part of the vagina to its lower part. This scanning was performed with a slice thickness of 8.0 mm and collimation of 4.0 mm. We used CT to reconstruct the images on three planes (upper, middle, lower level of the vagina) and measured the vertical space from the posterior wall of the vagina to the anterior wall of the rectum. We collected the measurement data and used *t*-test to analyze the results.

RESULTS

We measured the space between the posterior wall of the vagina and the anterior wall of the rectum of the volunteers at three levels (upper 1/3, middle, lower 1/3 level of the vagina) and collected data. The data was divided into three groups. The first group was measured at the upper 1/3 level of the vagina (Figure 1A), the second group was acquired at the middle level of the vagina (Figure 1B) and the third group was measured at the lower 1/3 level of the vagina (Figure 1C). The two-samples mean *t*-test was used to study differences between groups. Table 1 showed the data describing the distance between the posterior wall of the vagina and the anterior wall of the rectum at three levels (upper 1/3, middle, lower 1/3 level of the vagina). Table 2 presented the comparison of space size between the upper 1/3 level of the vagina and the middle level of the vagina and the comparison of space size between the middle level of the vagina and the lower 1/3 level of the vagina. Data was expressed as mean ± SD, and statistical significance was considered present when $P < 0.01$. In Table 2, the results showed the space from the posterior wall of the vagina to the anterior wall of the rectum was longer at the middle level of the vagina than at the upper 1/3 level of vagina ($P < 0.01$) and was shorter at the middle level of the vagina than at the lower 1/3 level of the vagina ($P < 0.01$).

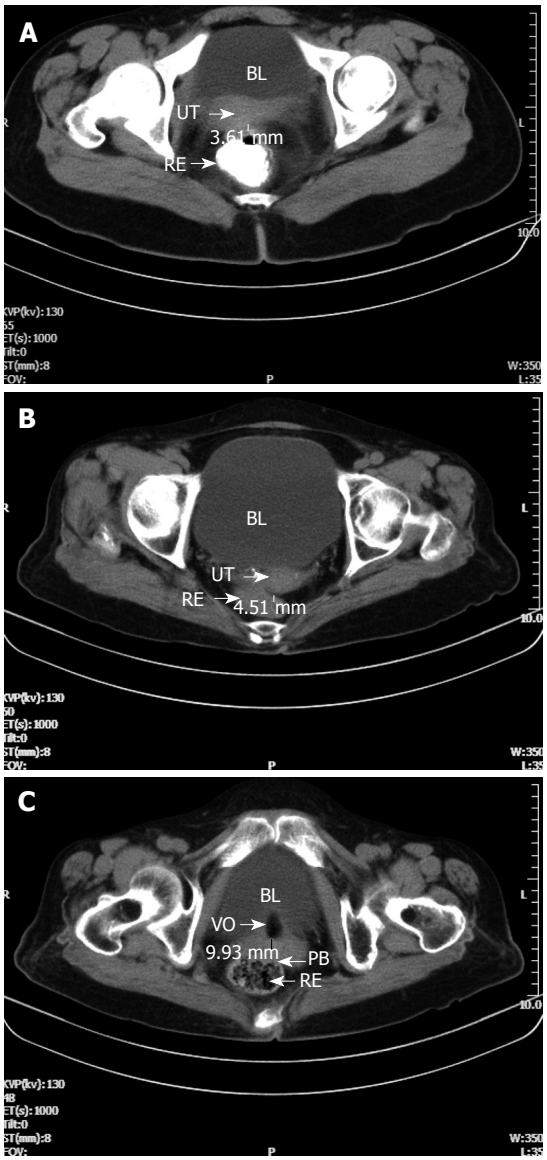

Figure 1 The space between the posterior wall of the vagina and the anterior wall of the rectum. A: Upper 1/3 level of the vagina; B: Middle level of the vagina; C: Lower 1/3 level of the vagina. BL: Bladder; UT: Uterus; RE: Rectum; VO: Vagina orifice; PB: Perineal body.

DISCUSSION

Rectocele is defined as herniation of the anterior rectal wall into the posterior vaginal wall. Rectoceles may be classified according to their position (low, middle, high); size (small < 2 cm, medium 2-4 cm, large > 4 cm); degree (type 1, type 2). The classifications of position and size are pure an anatomical description. However, the degree type could lead surgeons to make different decisions for management, which were proposed by Nichols and Pochak[1] and Pucciani[2]. Although it was a well-known fact that rectocele results from the weakness of the rectovaginal septum, there was a long-standing debate about the rectovaginal septum because of several early anatomical studies unable to determine the presence of rectovaginal septum. In 1970, Nichols *et al*[10] were able to identify a definitive anatomical and histological facial structure between the rectum and vagina in all dissections. Delancey[11,12] confirmed the fibers of rectovaginal septum ran vertically and blended with the muscular wall of the vagina. He also demonstrated the presence of some other posterior vaginal wall supporting structures which include the endopelvic facia, levator anti muscle and perineal membrane. In our study, we measured the distance between the posterior wall of the vagina and the anterior wall of the rectum (Tables 1 and 2). The results showed the distance becomes wider leading from top to bottom (Figure 1). The data also revealed that the rectovaginal septum becomes gradually thicker leading from top to bottom because the septum is the main portion between the gap from vagina to rectum. Based on these

Table 1 Data on the space between the vagina and the rectum at three levels (mm)

n	Group 1 (upper 1/3 level)	Group 2 (middle level)	Group 3 (lower 1/3 level)
n_1	3.61	4.51	9.93
n_2	4.02	4.87	10.14
n_3	3.34	4.92	10.26
n_4	4.31	4.38	10.38
n_5	3.29	4.65	9.87
n_6	4.04	4.73	9.66
n_7	3.83	4.89	9.54
n_8	3.91	4.99	10.23
n_9	3.96	5.08	9.66
n_{10}	4.26	4.16	9.78
n_{11}	3.73	4.26	9.88
n_{12}	4.26	4.34	9.46
n_{13}	2.96	4.67	9.83
n_{14}	4.15	4.54	10.23
n_{15}	3.86	4.78	10.49
n_{16}	4.11	4.96	10.57
n_{17}	4.29	5.21	11.04
n_{18}	4.16	4.17	10.87
n_{19}	3.97	4.49	9.38
n_{20}	3.86	4.55	9.96

Table 2 Comparison of the space difference at the upper 1/3 level of the vagina, the middle level of the vagina and the lower 1/3 level of the vagina

	Group 1 (n = 20)	Group 2 (n = 20)	Group 3 (n = 20)	*P* value
mean ± SD (mm)	3.896 ± 0.3617	4.6575 ± 0.3052	10.058 ± 0.4534	$P < 0.01$

measurement findings, it seemed that the higher position rectocele occurred more easily.

Although the true incidence of rectocele in the general population is uncertain, it has been found in 20%-80% of women referred to pelvic floor clinics[13]. The difficulties in diagnosis are not only because early rectocele is asymptomatic, but also because the dominant symptoms of rectocele are complex. Shorvon and colleagues[14] defined the rectocele depth exceeding 1 cm as pathological rectocele. At this stage, it is possible that patients have no sensation of difficulty in evacuation, constipation and so on. As a result , some early rectocele patients may be neglected. This study investigated the size of the normal space between the posterior wall of the vagina and the anterior wall of the rectum (Table 1). The study may be useful in the early diagnosis of rectocele by comparison of the patient's data with normal data.

The surgical indications for rectocele repair are controversial. Most surgeons advocated operative repair if the quality of life of the patient was affected, for instance they had a large symptomatic rectocele and failed to empty sufficiently. Surgery for rectocele repair included several different techniques using different approaches, ranging from the endorectal to the perineal or vaginal route[15,16]. Endorectal rectocele repair was developed by Sullivan[17]; it's advantages included an ability to deal with coincident anorectal pathology (in particular hemorrhoids and anterior mucosal rectal prolapse), with a definitive defect-specific septal repair and excision of the redundant rectal mucosa. The transperineal rectocele repair was described by Watson[18]; this method has been used to restore the anatomical pelvic floor structures and repair the rectovaginal septum. The traditional transvaginal approach was developed further into posterior colpoperineorrhaphy by Helgar. This surgery technique was used for all forms of genital and related rectal prolapse. However, this procedure destroyed the perineal body and created a tight band inside the vaginal introitus. We also observed the perineal body at the lower 1/3 level of the vagina (Figure 1C), which joined together the distal part of transvaginal septum. It is obvious that the whole perineal body is an important pelvic floor supporting structure. However, all these surgery techniques have been reported to produce some postoperative symptoms. For example, the difficult evacuation has been reported with endorectal repair. Transvaginal repair and transperineal repair have also been criticized because of sexual discomfort. A combination of the three surgery techniques was popularized recently. This produced a strong rectovaginal septum to avoid rectocele and also eliminated postoperative symptoms. Furthermore, the anatomical restoration of normal space between the rectum and vagina is key to resolving complex postoperative symptoms, e.g. difficult evacuation, sexual discomfort and so on. There is limited measurement data available relating to the space between the rectum and the vagina, which can be helpful for surgeons in assessing clinical diagnosis and functional outcomes of different types of rectocele repair. The present study provides measurement data on the normal space between rectum and vagina which may be useful for assessing clinical diagnosis of rectocele and functional outcomes of rectocele repair.

COMMENTS

Background

Rectocele is a common problem in females. In the past few decades, several techniques have been proposed for treating rectocele. However, it is difficult to diagnose rectocele early because there is no data on the normal size of the space between the rectum and vagina. There are several different techniques available for repair of rectocele, but they are associated with some postoperative symptoms. This study may be helpful for surgeons in assessing clinical diagnosis and functional outcomes of different types of rectocele repair.

Research frontiers

Surgery for rectocele repairs includes several different techniques using different approaches, ranging from the endorectal to the perineal or vaginal route. Nowadays a combination of the three surgery techniques is popular, which not only produces a strong rectovaginal septum to avoid rectocele but also eliminates postoperative symptoms.

Innovations and breakthroughs

This study revealed that the rectovaginal septum becomes gradually thicker going from top to bottom. Furthermore, based on these measurement findings, surgeons could assess clinical diagnosis and functional outcomes of different types of rectocele repair.

Terminology

Rectocele can be defined as herniation of the anterior rectal wall into the posterior vaginal wall.

Peer review

The manuscript is simple, but important for colorectal surgeons, gynecologists and general surgeons who daily manage this disease.

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Current use of immunosuppressive agents in inflammatory bowel disease patients in East China

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Abstract

AIM: To investigate immunosuppressive agents used to treat inflammatory bowel disease (IBD) in East China.

METHODS: A retrospective review was conducted, involving 227 patients with IBD admitted to Sir Run Run Shaw Hospital, College of Medicine, Zhejiang University from June 2000 to December 2007. Data regarding demographic, clinical characteristics and immunosuppressants usage were analyzed.

RESULTS: A total of 227 eligible patients were evaluated in this study, including 104 patients with Crohn's disease and 123 with ulcerative colitis. Among the patients, 61 had indications for immunosuppressive agents use. However, only 21 (34.4%) received immunosuppressive agents. Among the 21 patients, 6 (37.5%) received a subtherapeutic dose of azathioprine with no attempt to increase the dosage. Of the 20 patients that received immunosuppressive agent treatment longer than 6 mo, 15 patients went into remission, four patients were not affected and one relapsed. Among these 20 patients, four patients suffered from myelotoxicity and one suffered from hepatotoxicity.

CONCLUSION: Immunosuppressive agents are used less frequently to treat IBD patients from East China compared with Western countries. Monitoring immunosuppressive agent use is recommended to optimize dispensation of drugs for IBD in China.

Key words: Inflammatory bowel disease; Immunosuppressive agents; Azathioprine

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INTRODUCTION

Inflammatory bowel disease (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), is a chronic disorder of the gastrointestinal tract. There have been no large scale epidemiological studies on the incidence and prevalence of IBD in China, but reports indicate that rates are increasing. According to data collected from multiple hospitals, the prevalence rate of UC and CD can be speculatively estimated to be $11.6/10^5$ and $1.4/10^5$, respectively; however, the numbers may be underestimated[1]. An investigation from one hospital showed that the definitive cases of IBD during the past 10 years have increased five-fold[2].

Immunosuppressive agents, such as Azathioprine (AZA), play an essential role in drug therapy of IBD. Evidence-based medicine has shown that immunosuppressive agents can control active inflammation, allow for the withdrawal of steroids, and ultimately maintain long-term remission of IBD[3-5]. However, great interpatient variability has been found when assessing the efficacy and toxicity of these drugs. In the treatment of active disease, about 2/3 of the patients achieve remission, but this results is not achieved in approximately 15% of cases, and serious

drug toxicity leads to cessation of therapy in 9%-28% of patients, such as myelotoxicity and hepatotoxicity[6,7]. Uncertainty regarding the risk of interpatient variability and serious drug toxicity prevent the use of AZA and other immunosuppressants, and therefore affects the quality of care in IBD patients. Domestically, the paradox is quite a problem. Currently, there are no reports about the status of usage of AZA and other immunosuppressive agents in Chinese IBD patients.

Sir Run Run Shaw Hospital is a teaching hospital affiliated to Zhejiang University, China. Together with 12 other large hospitals and numerous smaller district hospitals, it provides health care to 48 million people living in Zhejiang Province in eastern China. Our IBD study group has set up an IBD database to collect IBD patients' data in East China. The purpose of the current study was to do a retrospective study of the therapeutic status of immunosuppressive agents, such as AZA used in patients with IBD in hospitals, to investigate the therapeutic implications of IBD in eastern China, to arouse the attention of clinicians, to apply immunosuppressive drugs optimally and to enhance the quality of therapy delivered to IBD patients.

MATERIALS AND METHODS

Subjects

IBD patients admitted to Sir Run Run Shaw Hospital, College of Medicine, Zhejiang University, from June 2000 to December 2007, were enrolled in this study.

The diagnosis of IBD was confirmed by the criteria established by Chinese Society of Gastroenterology[8] in 2000 and the guidelines issued by the Clinical Services Committee of the British Society of Gastroenterology (BSG)[9] in 2004. Patients not under the care of a gastroenterologist were excluded.

Data collection

A retrospective review was performed. Clinical data on demographic information, clinical characteristics of IBD patients, as well as endoscopic, radiologic, surgical and pathological records, confirmed diagnoses, duration and severity of disease, and use of immunomodulatory agents were collected from the inpatient and follow-up clinic visit records and collated in an IBD database.

Statistical analysis

The data were expressed as mean values, and the enumeration data were expressed by percentages. All statistical analyses were performed by SPSS V 13.0 (Statistical Product and Service Solutions).

RESULTS

Patient characteristics

According to the inclusion and exclusion criteria, 104 patients with CD and 123 patients with UC were enrolled in the study. All the patients were Han Chinese.

UC was categorized by extent, activity and severity of disease. Extent of disease at diagnosis was defined macroscopically by the proximal limit of inflammation at colonoscopy and was divided into the following four categories: (1) Proctitis, inflammation confined to the rectum only; (2) Distal colitis, inflammation involving to the rectum and sigmoid colon; (3) Left-sided colitis, inflammation extending the rectum to and including the splenic flexure; (4) Extensive colitis, inflammation proximal to the splenic flexure.

Patients with Crohn's disease were classified by age of onset, disease location and behaviour according to the Vienna classification[10]. Disease activity was assessed using the Harvey-Bradshaw index (HBI) for CD and the Sutherland index for UC. Active disease was defined as a HBI value $\geqslant$ 5 or a Sutherland index $\geqslant$ 3. Severity was broadly divided into mild, moderate or severe according to the Truelove & Witts' criteria for UC and the criteria established by the Chinese Society of Gastroenterology for CD.

Severe UC was defined as the passage of $\geqslant$ 6 bloody stools daily with one or more of the following criteria: temperature > 37.8℃, pulse > 90/min, haemoglobin < 10.5 g/dL, or erythrocyte sedimentation rate (ESR) > 30 mm/h[11].

Mild CD was defined when the patient had no fever, abdominal tenderness, abdominal mass and obstruction, while severe CD was defined when the patient had persistent high fever, weight loss, nausea, vomiting, abdominal pain, diarrhea, anemia, and complications. Characteristics of the patients and the information about the location of disease are given in Table 1.

Table 1 Clinical characteristics of the study population

	Crohn's disease	Ulcerative colitis
Number of patients	104	123
Female	42 (40.4%)	56 (45.5%)
Age (yr)	36 (13-70)	45 (15-80)
Disease duration (yr)	4.8 (0.5-24)	5.5 (0.5-23)
Non-smoking	82 (78.8%)	90 (73.2%)
Severity, *n* (%)		
Mild	24 (23.1)	62 (50.4)
Moderate	51 (49)	42 (34.1)
Severe	29 (27.9)	19 (15.5)
Disease distribution, *n* (%)		
Small intestine	44 (42.3)	
Ileum and colon	17 (16.3)	
Colon	40 (38.5)	
Upper digestive tract	3 (2.9)	
Disease distribution (UC), *n* (%)		
Distal colon		41 (33.3)
Left side colon		24 (19.5)
Systemic or pan-colon		58 (47.2)
History of any intestinal operation	46 (44.2)	5 (4.1)

Of the 227 patients, 61were administered immunosuppressant agents. Among these patients 27 (44.3%) were steroid-dependent (refers to a relapse when the steroid dose is reduced below 20 mg/d, or within 6 wk of stopping steroids), 6 (9.8%) were steroid-refractory (refers to active disease in spite of an adequate dose and duration of prednisolone > 20

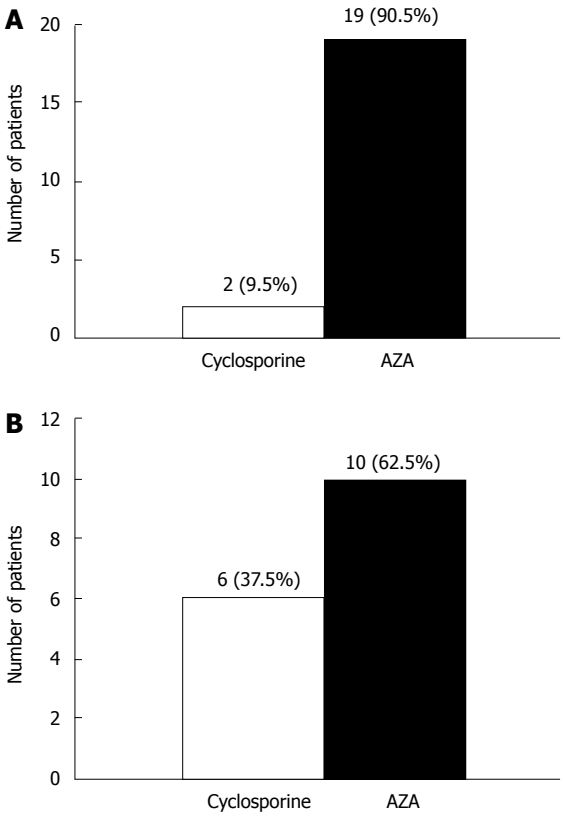

Figure 1 Drug therapeutic status of IBD patients who received immunosuppressant therapy. A: Twenty one IBD patients; B: AZA therapeutic status of 16 IBD patients.

mg/d for more than 2 wk) and 28 (45.9%) had post-surgery fistulating CD. Twenty-one patients (21/61, 34.4%) received immunosuppressive agents (19 cases with AZA, two cases with cyclosporin) as shown in Figure 1A. The mean dose of AZA was 1.47 mg/kg per day (range 0.83-2.22 mg/kg per day). A suboptimal dose of AZA was defined as a dose less than 1.5 mg/kg per day in the absence of myelotoxicity or hepatotoxicity. AZA-related myelotoxicity is defined as WBC $< 3.0 \times 10^9$/L or neutrophil $< 1.5 \times 10^9$/L, while AZA-related hepatotoxicity is defined as ALT and/or GGT levels greater than 5 times the upper normal limit, or ALP levels greater than 3 times the upper normal limit, excluding viral hepatitis. When withdrawing AZA or reducing the dose, the above indexes recover to normal. According to this criterion, among the 19 patients treated with AZA, there were two with myelotoxicity and one with hepatotoxicity, before the clinician adjusted the dosage. The other 16 residual cases received AZA therapy, and six of those cases (37.5%) received subtherapeutic therapy, with no attempt to increase this dosage (Figure 1B).

Therapeutic circumstances and adverse reactions in patients receiving immunosuppressant therapy

Of the 21 patients administered immunosuppressant therapy, 21 patients maintained these regimen for more than 6 mo, and one patient withdrew from drug without a recommendation by a clinician 3 mo later.

The effectiveness of the immunosuppressant therapy (disease remission) is defined as follows: for Crohn's disease, the HBI value was less than five or for UC the Sutherland index was less than three. According to this definition, of the 20 patients who received immunosuppressant therapy for more than 6 mo, 15 (75%) patients went into remission, four (20%) patients had no benefit and one (5%) relapsed.

According to the definitions of AZA-related myelotoxicity and hepatotoxicity, four (20%) patients suffered from myelotoxicity (two cases occurred within 3 mo after receiving AZA therapy; one case each occurred one and two years after drug therapy) and one (5%) patient suffered from hepatotoxicity.

DISCUSSION

IBD, which includes CD and UC, is a complicated disease of the digestive tract. In recent years, because of the rapid development of evidence-based medicine, the therapy guidelines of IBD in China and abroad have been updated constantly. Reddy *et al*[12] analyzed the therapeutic condition of American patients with IBD according to the American College of Gastroenterology (ACG) practice guidelines[13,14]. They found that the dose of aminosalicylic acid agents was not adequate and enema therapy and immunosuppressive agents were not applied effectively[13,14]. With the lack of relative data in China, we carried out this study to reflect the current therapeutic condition of patients with IBD in eastern China.

Although glucocorticoids are effective in the induction of remission for IBD, more than 20% of patients may be steroid-refractory or become steroid-dependent[15-17]. Meta-analyses showed that glucocorticoids are not effective for medical maintenance. The frequency and severity of well-recognized adverse effects also preclude their long-term use.

Immunosuppressive agents such as AZA can induce and maintain remission of IBD and have steroid sparing effects in patients who are steroid dependent or who have refractory IBD[7,18-20].

In our study, the use of immunosuppressive agents was restricted to a minority of IBD patients (19.6%) who adapted to use these drugs, which is distinctly less frequent compared with that in Western countries. Furthermore, more than half of the patients did not receive recommended doses of AZA. We found that the percentage of serious drug toxicity was 5/20 in AZA therapy. Because of the lack of data from large-scale studies in China, the adverse reaction rate of AZA in Han nationality Chinese with IBD is not clear. Results of our study are consistent with the conclusions reported outside of China[21].

At present, many researchers presume that interpatient and interracial variability, which are based on thiopurine methyltransferase (TPMT) gene polymorphisms and enzyme activity, exist and affect the efficacy and toxicity of AZA. Whether TPMT gene

polymorphisms and enzyme activity have their own characteristics in this Han Chinese population, and how they affect the efficacy and toxicity of drug still remains to be seen. Because large-scale studies in China are not available, we do not have relevant data about TPMT gene polymorphisms and enzyme activity, relative studies about the efficacy and toxicity of drugs when given with TPMT, relevant screening methods for high risk groups before using drug or relative monitoring methods for the efficacy and toxicity of drug. Serious drug toxicities, such as myelotoxicity and hepatotoxicity, deter the use and prevent achieving the optimal dose of immunosuppressive agents by clinicians. Experience abroad has shown that AZA can be safely used for treatment of IBD[22-24].

Recently, TPMT and its genotype have been applied to predict the efficacy and toxicity of drugs, which can provide some guidance for clinicians and reduce the incidence of drug toxicity in Western countries[25-29]. Meanwhile, studies have shown that examining TPMT enzyme activity may decrease the overall medical cost in Europe and America[30,31].

Therefore, developing a study of TPMT polymorphisms and enzyme activity in Han nationality Chinese with IBD and clarifying the correlation between the efficacy and toxicity of drugs and TPMT will provide theoretical evidence for the clinical application of immunosuppressive agents, such as AZA. Developing techniques to examine TPMT polymorphisms and enzyme activity, as well as concentrations of AZA's metabolites, will be helpful to screen patients in high risk groups, reduce the incidence of toxicity because of drugs, improve the rationality and reliability of pharmacotherapy, setup an individualization of therapeutic schedules, and broaden the therapeutic modalities for IBD patients.

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COMMENTS

Background

Immunosuppressive agents are well established in the treatment of inflammatory bowel disease. The goals of using this class of medication are to control active inflammation, allow for the withdrawal of steroids, and ultimately to maintain long-term remission of inflammatory bowel disease (IBD).

Research frontiers

Immunosuppressive agents, such as Azathioprine (AZA), play an essential role in the pharmacotherapy of IBD. Evidence-based medicine proved that immunosuppressive agents such as AZA can induce and maintain remission of IBD and have steroid sparing effects in patients who are steroid dependent or who have refractory IBD. However, experience abroad has shown great interpatient variability in the efficacy and toxicity of these drugs.

Innovations and breakthroughs

The use of immunosuppressive agents in IBD patients in China has not been reported. The authors designed this study to address this problem and provide data to improve the quality of IBD treatment in China.

Peer review

This is an important study and interesting topic, especially for an Asian readership.

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BRIEF ARTICLES

Hepatic injury induced by carbon dioxide pneumoperitoneum in experimental rats

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Abstract

AIM: To observe the hepatic injury induced by carbon dioxide pneumoperitoneum in rats and to explore its potential mechanism.

METHODS: Thirty healthy male SD rats were randomly divided into control group (n = 10), 0 h experimental group (n = 10) and 1 h experimental group (n = 10) after sham operation with carbon dioxide pneumoperitoneum. Histological changes in liver tissue were observed with hematoxylin-eosin staining. Liver function was assayed with an automatic biochemical analyzer. Concentration of malonyldialdehyde (MDA) and activity of superoxide dismutase (SOD) were assayed by colorimetry. Activity of adenine nucleotide translocator in liver tissue was detected with the atractyloside-inhibitor stop technique. Expression of hypoxia inducible factor-1 (HIF-1) mRNA in liver tissue was detected with *in situ* hybridization.

RESULTS: Carbon dioxide pneumoperitoneum for 60 min could induce liver injury in rats. Alanine aminotransferase and aspartate aminotransferase were 95.7 ± 7.8 U/L and 86.8 ± 6.9 U/L in 0 h experimental group, and 101.4 ± 9.3 U/L and 106.6 ± 8.7 U/L in 1 h experimental group. However, no significant difference was found in total billirubin, albumin, and pre-albumin in the three groups. In 0 h experimental group, the concentration of MDA was 9.83 ± 2.53 μmol/g in liver homogenate and 7.64 ± 2.19 μmol/g in serum respectively, the activity of SOD was 67.58 ± 9.75 nu/mg in liver and 64.47 ± 10.23 nu/mg in serum respectively. In 1 h experimental group, the concentration of MDA was 16.57 ± 3.45 μmol/g in liver tissue and 12.49 ± 4.21 μmol/g in serum respectively, the activity of SOD was 54.29 ± 7.96 nu/mg in liver tissue and 56.31 ± 9.85 nu/mg in serum, respectively. The activity of ANT in liver tissue was 9.52 ± 1.56 in control group, 6.37 ± 1.33 in 0 h experimental group and 7.28 ± 1.45 (10^{-9} mol/min per gram protein) in 1 h experimental group, respectively. The expression of HIF-1 mRNA in liver tissue was not detected in control group, and its optical density difference value was 6.14 ± 1.03 in 0 h experimental group and 9.51 ± 1.74 in 1 h experimental group, respectively.

CONCLUSION: Carbon dioxide pneumoperitoneum during the sham operation can induce hepatic injury in rats. The probable mechanisms of liver injury include anoxia, ischemia reperfusion and oxidative stress. Liver injury should be avoided during clinical laparoscopic operation with carbon dioxide pneumoperitoneum.

Key words: Carbon dioxide pneumoperitoneum; Hepatic injury; Rat; Anoxia; Laparoscopic operation

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INTRODUCTION

Along with the utilization of laparoscope in surgery,

more and more patients can recover with less injuries and complications. However, laparoscopic operation is always limited due to exposure of the organs. Although carbon dioxide pneumoperitoneum is a desirable method to assist in exposing abdominal organs, the high pressure of carbon dioxide in abdominal cavity has some potential side effects, such as impairment of liver, kidney and heart functions[1-3]. Some researches revealed that the continuous high pressure from carbon dioxide during laparoscopic operation can result in ischemia injury in multiple organs, and the longer the operation lasts, the severer the injury is[2]. One of the important reported mechanisms of liver injury is ischemia reperfusion[1,2]. This study was to observe whether sham operation with carbon dioxide pneumoperitoneum causes liver injury and to explore its probable mechanism.

MATERIALS AND METHODS

Animals

Thirty healthy male SD rats were randomly divided into control group (n = 10), 0 h experimental group (n = 10), and 1 h experimental group (n = 10) after sham operation with carbon dioxide pneumoperitoneum. All experimental rats received sham operation for 1 h. Rats in the two experimental groups accepted carbon dioxide pneumoperitoneum during operation. The pressure of carbon dioxide was 15 mmHg. Liver tissue and serum were collected for further test.

Reagents

Oligo-nucletide probe of hypoxia inducible factor 1 (HIF-1) mRNA was produced by Shanghai Shenneng Biotechnology Company (China). ^{3}H-ADP and atractyloside (ATR) were obtained from Sigma Company (USA).

Methods

All rats were anaesthetized with pentobarbital sodium muscular injection. Rats in the two experimental groups received carbon dioxide pneumoperitoneum for 1 h during sham operation. Rats in the control group only underwent sham operation for 1 h. Blood samples and liver tissues were taken immediately from rats in 0 h experimental group and control group, and from rats in 1 h experimental group after sham operation, respectively. Liver function was detected with an automatic biochemistry analyzer. Histological changes in liver tissue were observed with hematoxylin-eosin (HE) staining under optical microscope. Concentration of malonyldialdehyde (MDA) in liver homogenate and serum was measured by thio-barbituric acid colorimetry using a spectrophotometer at the wave length of 532 nm and expressed as μmol/g. Activity of superoxide dismutase (SOD) was detected by xanthine oxidase colorimetry and expressed as nu/mg. Mitochondria in liver tissue were isolated by centrifugation. Activity of ANT in liver tissue was detected with the ATR-inhibitor stop technique. Mitochondria were initiated by adding ^{3}H-ADP and terminated after 12 s by adding ADR. Radioactivity in each group was measured and activity of ANT was expressed as 10^{-9} mol/min per gram protein. Expression of HIF-1 mRNA in liver tissue was detected with *in situ* hybridization (ISH). The results of ISH were quantified with an electronic computer and shown as absorbance (A) value.

Statistical analysis

Experimental data were expressed as mean ± SD. All data were analyzed by t test using SPSS 10.0 statistical software.

RESULTS

Liver function

Liver function in the two experimental groups was disturbed obviously compared with the control group (Table 1). After sham operation with carbon dioxide pneumoperitoneum, the level of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) was 86.8 ± 6.9 U/L and 95.7 ± 7.8 U/L respectively in 0 h experimental group A, which was higher than that in control group (P < 0.05). The level of AST and ALT was 106.6 ± 8.7 U/L and 101.4 ± 9.3 U/L respectively in 1 h experimental group, which was higher than that in control group (P < 0.05). No significant difference was observed in the levels of total bilirubin (TB), albumin (A) and pre-albumin (Pre-A) between the two experimental groups.

MDA concentration and SOD activity in liver homogenate and serum

In control group, the concentration of MDA in liver homogenate and serum was 4.69 ± 1.31 μmol/g and 3.98 ± 1.05 μmol/g, respectively. After sham operation with carbon dioxide pneumoperitoneum, the concentration of MDA in liver homogenate and serum was significantly elevated in the two experimental groups (Table 2), indicating that liver is more susceptible to hypoxia injury. The activity of SOD in control group was 80.56 ± 12.43 nu/mg in liver homogenate and 75.66 ± 11.35 nu/mg in serum, respectively. The activity of SOD in liver homogenate and serum was significantly decreased in the two experimental groups (P < 0.05), demonstrating that more oxygen radicals are produced in the two experimental groups and SOD is consumed after elimination of oxygen radicals.

Histological changes in liver tissue

Liver samples were embedded in paraffin, stained with HE and then observed under an optical microscope. The livers of control group showed a normal lobular architecture with central veins and radiating hepatic cords, indicating that sham operation with routine anesthesia does not cause histopathological damage to liver. Mild hepatic fatty degeneration and necrosis were found in livers of the two experimental groups

Table 1 Changes in liver function (mean ± SD)

Group	TB (μmol/L)	ALT (U/L)	AST (U/L)	A (g/L)	Pre-A (mg/L)
Control	24.5 ± 5.1	48.5 ± 8.2	45.6 ± 7.7	38.5 ± 4.2	203.5 ± 76.4
0 h experimental group	23.1 ± 3.7	95.7 ± 7.8[a]	86.8 ± 6.3[a]	37.4 ± 3.1	195.8 ± 41.5
1 h experimental group	25.8 ± 3.5	101.4 ± 9.3[a]	106.6 ± 8.7[a]	40.6 ± 3.9	182.9 ± 58.4

[a]$P < 0.05$ *vs* control group.

Table 2 MDA concentration and SOD activity in liver homogenates and serum (mean ± SD)

Group	MDA (liver) (μmol/g)	SOD (liver) (nu/mg)	MDA (serum) (μmol/g)	SOD (serum) (nu/mg)
Control group	4.69 ± 1.31	80.56 ± 10.43	3.98 ± 1.05	75.66 ± 9.35
0 h experimental group	9.83 ± 2.23[a]	67.58 ± 9.75[a]	7.64 ± 2.39[a]	64.47 ± 10.23[a]
1 h experimental group	16.57 ± 3.45[a]	54.29 ± 7.96[a]	12.49 ± 4.21[a]	56.31 ± 9.87[a]

[a]$P < 0.05$ *vs* control group.

Table 3 Activity of ANT in mitochondria of liver (mean ± SD)

Group	ANT (10^{-9} mol/min per gram protein)
Control group	9.52 ± 1.76
0 h experimental group	6.37 ± 1.23[a]
1 h experimental group	7.21 ± 1.05[a]

[a]$P < 0.05$ *vs* control group.

(Figure 1A). The hepatic injury was more severe in 1 h experimental group B than in 0 h experimental group, suggesting that carbon dioxide pneumoperitoneum can cause hepatic injury (Figure 1B).

Activity of ANT in mitochondria of liver

In control group, the activity of ANT was 9.52 ± 1.56 (10^{-9} mol ADP/min per gram protein). In 0 h experimental group A, it was only 6.37 ± 1.33 ($P < 0.05$ compared with control group), indicating that energy metabolism in mitochondria of liver is damaged by carbon dioxide pneumoperitoneum. One hour after carbon dioxide pneumoperitoneum, the activity of ANT was slightly increased (7.21 ± 1.05) compared with control group ($P < 0.05$). However, it was lower in 1 h experimental group than in control group, indicating that energy metabolism in mitochondria of liver is recuperated to some extent after sham operation with carbon dioxide pneumoperitoneum (Table 3).

Expression of HIF-1 mRNA in liver tissue

No expression of HIF-1 mRNA was found in liver tissue from control group, indicating that sham operation without carbon dioxide pneumoperitoneum does not cause hypoxia stimulation in liver (Figure 2A). The expression of HIF-1 mRNA was significantly increased in the two experimental groups. The *A* value for HIF-1 mRNA was 6.14 ± 1.03 in 0 h experimental group and 9.51 ± 1.74 in 1 h experimental group ($P < 0.05$). Brown positive particles of HIF-1 mRNA, mainly located in cytoplasm of liver cells, were more in stromal cells than in hepatocytes (Figure 2B). Whether the stromal cells are hepatic stellate cells or endotheliocytes remains unknown. The expression of HIF-1 mRNA was increased more significantly in 1 h experimental group compared with 0 h experimental group, indicating that there exists persistent hypoxia stimulation in liver after carbon dioxide pneumoperitoneum (Figure 2C).

DISCUSSION

Laparoscopic operation, performed frequently in recent years, has many advantages over conventional surgery, such as less injuries and complications. Thus patients who accept laparoscopic operation can recover with a shorter healing time and less operative scars. However, exposure of organs is always not enough. Carbon dioxide pneumoperitoneum is a desirable method to assist in exposing abdominal organs. Some researches have shown that it has some potential side effects, such as impairment of liver, kidney, and heart functions[1-4]. It has been reported that the continuous high pressure from carbon dioxide during laparoscopic operation can result in ischemia injury of multiple organs, and the longer the operation lasts, the severer the injury is[5,6].

In this study, 1 h after sham operation with carbon dioxide pneumoperitoneum, the serum ALT and AST levels were increased while the levels of TB, A and Pre-A were not significantly changed. Since the half life of albumin is 14 d, the reduced albumin level can demonstrate the chronically impaired synthetic function of liver. However, the half life of prealbumin is only 2 d, and accordingly, a low level of prealbumin in serum indicates acute impairment of liver synthetic function. This study showed that liver function injury was not severe enough to cause hypoproteinemia. However, more susceptible markers of the liver function, ALT and AST, demonstrated mild impairment of liver function. It has been shown that necrosis of even a few hepatocytes results in a high level of transaminase[7-10]. In HE stained liver samples, fatty degeneration was found in some hepatocytes, indicating that ischemia or anoxia occurs during sham operation with carbon

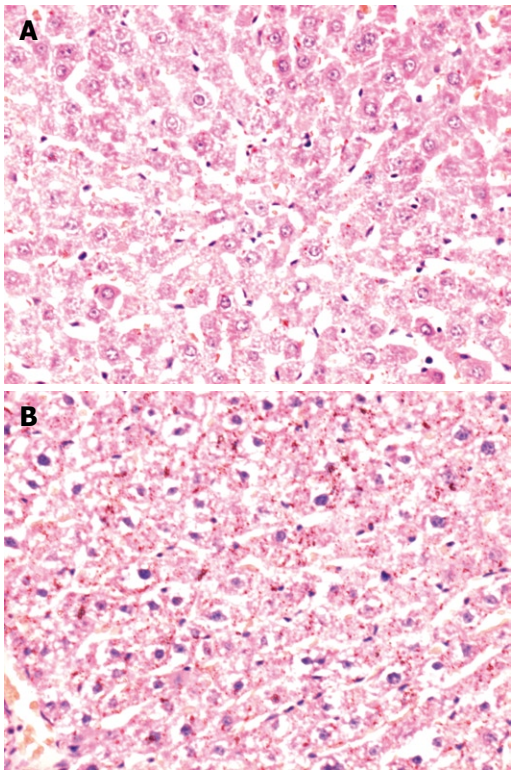

Figure 1 Liver injury in 0 h experimental group (A) and 1 h experimental group (B).

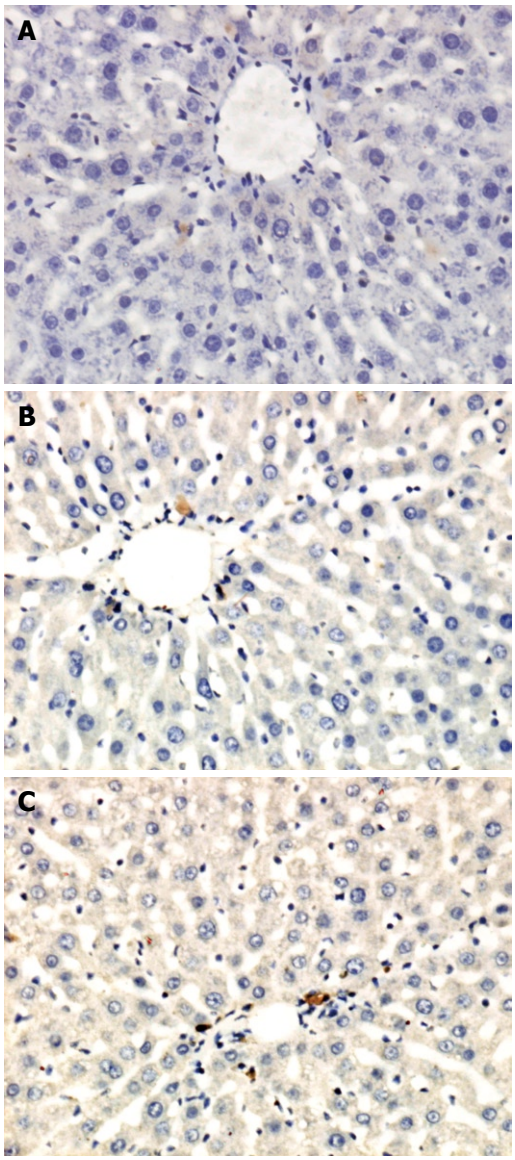

Figure 2 Expression of HIF-1 mRNA in liver tissue from control group (A), 0 h experimental group (B), and 1 h experimental group (C).

dioxide pneumoperitoneum[11]. The elevated expression level of HIF-1 mRNA in liver homogenate further indicates that anoxia injury is induced by carbon dioxide pneumoperitoneum. MDA is the end product of lipid peroxidation. The concentration of MDA in liver homogenate or serum is a direct marker for the level of oxygen radicals. SOD is one of the important scavenger enzymes of oxygen radicals. The activity of SOD would decrease after oxygen radicals are cleaned. In this study, the concentration of MDA was elevated and the activity of SOD was reduced in liver homogenate and serum, indicating that the number of oxygen radicals is increased after carbon dioxide pneumoperitoneum. Since liver is a motochondria-abundant organ, it is more susceptible to hypoxia than other organs. In this study, the activity of ANT, a marker of energy metabolism in mitochondria[12], was reduced after carbon dioxide pneumoperitoneum. The activity of ANT was mildly elevated 1 h after carbon dioxide pneumoperitoneum compared with the control group. It has been shown that blood-supply is obviously decreased in portal vein during carbon dioxide pneumoperitoneum[13]. In addition, hypercapnemia is related to ischemia injury of abdominal organs, while high pressure during operation and immediate relief of carbon dioxide after operation can induce ischemia reperfusion injury of multiple organs and apoptosis of hepatocytes after carbon dioxide pneumoperitoneum[14,15]. In this study, hepatic injury in rats and the possible mechanism of carbon dioxide pneumoperitoneum were elicited. Since pathophysiological changes in rats are not always identical as those in human beings, injury of carbon dioxide pneumoperitoneum should be observed closely in clinical practice. Although impairment of liver function usually does not cause severe complications, it should be alleviated or avoided especially in patients with preceding liver diseases during sustaining laparoscopic operation[16-20]. It has been shown that liver injury is pressure-dependant[21]. The pressure of carbon dioxide used in pneumoperitoneum is 10-12 mmHg, which is higher than that (7-10 mmHg) in the port system[22]. The higher pressure from carbon dioxide influences the systemic and portal blood flow dynamics[23-26], and causes apoptosis of hepatocytes[27,28]. A shorter time or a lower carbon dioxide pressure in pneumoperitoneum might help to alleviate liver injury. Stepwise increasing carbon dioxide insufflation might also be an ischemic preconditioning method to reduce liver injury[29]. Further study is needed on the precise mechanism of carbon dioxide pneumoperitoneum and more effective methods should be found to avoid liver injury[30].

COMMENTS

Background

Along with the utilization of laparoscope and carbon dioxide pneumoperitoneum in surgery, potential side effects of carbon dioxide pneumoperitoneum have been noticed in recent years.

Research frontiers

Some researches have shown that carbon dioxide pneumoperitoneum can induce impairment of liver, kidney, and heart functions. Apoptosis of hepatocytes was observed in this study.

Innovations and breakthroughs

This study showed mild injury of liver function in rats. At the same time, the expression level of hypoxia inducible factor-1 mRNA in liver tissue was increased as an evidence of anoxia in liver.

Peer review

This describes liver injury caused by carbon dioxide pneumoperitoneum in experimental rats. Although the injury does cause significant complications in rats, close attention should be paid to hepatic injury or other injury in clinical practice. The results of this study are of practical values.

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CASE REPORT

Palliative cardia resection with gastroesophageal reconstruction for perforated carcinoma of the gastroesophageal junction

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Abstract

Iatrogenic perforation of esophageal cancer or cancer of the gastroesophageal (GE) junction is a serious complication that, in addition to short term morbidity and mortality, significantly compromises the success of any subsequent oncological therapy. Here, we present an 82-year-old man with iatrogenic perforation of adenocarcinoma of the GE junction. Immediate surgical intervention included palliative resection and GE reconstruction. In the case of iatrogenic tumor perforation, the primary goal should be adequate palliative (and not oncological) therapy. The different approaches for iatrogenic perforation, i.e. surgical versus endoscopic therapy are discussed.

Key words: Esophageal cancer; Esophageal perforation; Emergency surgery; Stent therapy

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INTRODUCTION

Iatrogenic perforation of cancer of the esophagus or the gastroesophageal (GE) junction is a potentially life-threatening complication. Its incidence has increased most likely because of more aggressive palliative endoscopic therapy[1], and the current widespread use of endoscopic ultrasound (EUS) for accurate preoperative staging[2]. Therapy and management, i.e. conservative versus surgical treatment remains controversial, with successful early outcome being described for both approaches[3,4]. Irrespective of the treatment, iatrogenic (or spontaneous) perforation of the tumor has been shown to be a strong negative predictive factor for long-term survival. Therapy should therefore focus on the immediate and efficient control of the perforation (such as drainage, stenting or resection), and on a satisfactory quality of life rather than on oncologically adequate treatment.

CASE REPORT

An 82-year-old man was referred to our department with perforation of a subtotal stenosing adenocarcinoma of the GE junction. Previous symptoms were vomiting and weight loss of 6 kg in the last 6-8 wk. In the initial computed tomography (CT) scan, no signs of distant metastases were present. The patient had a history of tuberculosis 40 years ago, and CT revealed massive pleural calcifications. He was on oral anticoagulation therapy because of paroxysmal supraventricular tachycardia. To complete the staging, EUS was performed after endoluminal dilation of the tumor and passage into the stomach. EUS demonstrated an uT3 stage with suspicious lymph nodes. Following the EUS procedure, the patient developed severe abdominal pain. Subsequent CT showed air in the distal mediastinum, as well as in the retroperitoneal and intraperitoneal space (Figure 1A and B). After referral, the patient presented in a reduced general condition with acute abdomen, and signs of sepsis (tachycardia, hypotension and tachypnea). Infection signs were slightly increased: C-reactive protein 0.6 mg/dL (normal < 0.5 mg/dL), leukocytes 11.0 G/L (normal 4-9 G/L). As a result of the clinical symptoms, free intra-abdominal air, and subtotal stenosing tumor, we decided against initial endoscopic intervention and for immediate

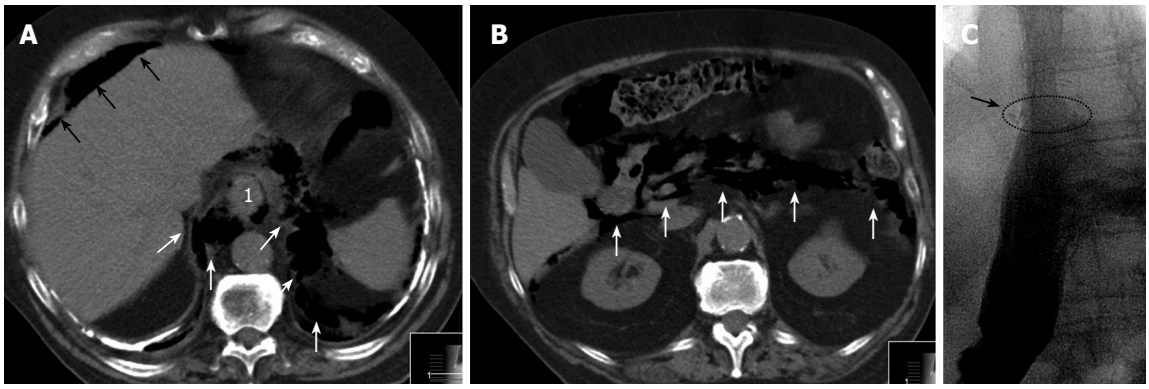

Figure 1 Pre- and postoperative radiographs. A and B: CT scan of the abdomen revealing free intra-abdominal air (A, black arrows) and massive mediastinal (A, white arrows) and retroperitoneal (B, white arrows) air. [1]Depicts the tumor localization at the GE junction; C: Water-soluble contrast medium swallowed after GE reconstruction (end-to-side esophago-gastrostomy) without evidence of anastomotic stenosis or leakage. The circle (arrow) points to the level of the anastomosis.

explorative laparotomy (10 h after the EUS procedure).

Intraoperatively, the tumor was localized exactly at the GE junction, with a dorsal perforation just proximal to the tumor. The tumor was removed completely by resection of the cardia and 5 cm of the distal esophagus. For reconstruction, a partial proximal gastric tube was constructed (20-25 cm in length with a diameter of 4-5 cm) using linear staplers (50 mm Proximate Linear Cutter; Ethicon Endo-Surgery, Inc., Cincinnati, OH, USA). The gastric antrum was opened from the ventral aspect and a circular stapler (25 mm CEEA circular stapler; Covidien Autosuture, Mansfield, MA, USA) was introduced to anastomose the distal esophagus with the proximal ventral portion of the gastric tube. Since the small bowel mesentery was rather short and not mobile, we decided against total gastrectomy and esophagojejunal reconstruction in this emergency situation. An oncological lymph node dissection was not carried out in this elderly and multimorbid patient.

The postoperative course was uneventful. The patient received intravenous antibiotics (imipenem/cilastatin, 3 × 500 mg/d), but no antifungal agent, for 7 d. A water-soluble contrast medium swallow on postoperative day 7 showed no signs of stenosis or anastomotic insufficiency (Figure 1C). The patient was put on full diet following this examination, and was discharged on postoperative day 14. On a further follow-up visit after 4 wk, the patients did not complain of reflux or dysphagic symptoms. Final histopathological examination revealed a perforated pT3 adenocarcinoma of the GE junction. The case was discussed interdisciplinarily, and no additive/palliative therapy was initiated because of the low WHO performance status of 2-3.

DISCUSSION

Perforation in patients with potentially curative resectable cancer of the esophagus or GE junction reduces dramatically the chance of long-term survival. Immediate therapy should target the potential septic focus either by drainage and stenting the lesion, or by resection. Secondary considerations include oncologically adequate treatment and reconstruction of the GE passage that offers the best quality of life.

The approach of conservative versus surgical therapy in cases of iatrogenic perforation has shifted more towards conservative therapy, together with the development of novel endoscopic stenting possibilities[1]. The question of whether iatrogenic perforations are best managed by surgery or endoscopy has recently been addressed by two large studies. Di Franco *et al*[5] have examined 48 patients with iatrogenic perforation of esophageal cancer. Sixteen patients were treated by oncological esophagectomy, and 32 were treated conservatively because of advanced disease in 17 and poor performance status in 15. The authors demonstrated that all patients in the resection group died of recurrent disease and more than half of them died within the first year after surgery. The difference in survival between the resected and non-resected group of patients was not significant. Similarly, Jethwa *et al*[6] have analyzed 83 iatrogenic perforations during diagnostic endoscopy, of which, 27 were managed by surgery. The median survival in the whole cohort was 72 d. There was a trend for longer survival in patients undergoing surgery. However, the high 30-d mortality of nearly 40% and the poor survival in the surgical and non-surgical group shows that even rapid surgical treatment often fails to change the natural course of the disease at this stage. Together, both studies suggest that the primary approach to perforated esophageal cancer should be conservative.

However, under certain conditions, the conservative approach is not feasible; e.g., the perforation is too extensive for adequate stent therapy, or, as in our case, the tumor is (subtotal) stenosing, making successful stent therapy exceedingly difficult. Other indications for a surgical approach include extensive peritonitis or mediastinitis that cannot be drained adequately by interventional drainage placement. Irrespective of the indication for surgery, it should entail the least invasive measure that offers the greatest chance of immediate survival and the best quality of life for the remaining time period. Thus, whenever possible, the esophago-intestinal continuity should be re-established.

In the present case, we opted for reconstruction using an end-to-side esophago-gastrostomy. Limited resection of the cardia and the distal esophagus has been described

particularly for early tumors of the GE junction. While this procedure is safe and effective, and does not seem to result in postgastrectomy symptoms or microgastria[7], other reports have highlighted the long-term risk of reflux esophagitis when using esophagogastrostomy for reconstruction[8]. Other options include the Merendino procedure or total gastrectomy with esophago-jejunostomy reconstruction[9]. However, while the former one would seem too time consuming and technically demanding in an emergency situation[8], the latter requires an adequate mobile jejunum, and there is evidence of an early clinical benefit from formation of a gastric reservoir[9].

In conclusion, the management of esophageal perforation in the context of an underlying malignancy demands an individual approach that depends upon the site and etiology of the perforation. Irrespective of the therapeutic approach, the prognosis after tumor perforation is dismal. Therefore, the best palliative procedure has to be chosen, which is in most instances, a conservative one with drainage and stenting, or limited surgery with re-establishment of the esophago-intestinal continuity.

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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
London, UK
Gastro 2009 UEGW/World Congress of Gastroenterology
www.gastro2009.org

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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Instructions to authors

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Acknowledgments

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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 Xiaohua Zazhi* 1999; **7**: 285-287

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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 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 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]

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9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

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10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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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

Author(s) and editor(s)

12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

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 ± SD or mean ± SE.

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Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

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Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.
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