ISSN 1007-9327
CN 14-1219/R

# World Journal of Gastroenterology®

**Indexed and Abstracted in:**

Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®) and Journal Citation Reports/Science Edition, *Index Medicus*, MEDLINE and PubMed, Chemical Abstracts, EMBASE/Excerpta Medica, Abstracts Journals, *Nature Clinical Practice Gastroenterology and Hepatology*, CAB Abstracts and Global Health.
ISI JCR 2003-2000 IF: 3.318, 2.532, 1.445 and 0.993.

**Volume 15 Number 17**
**May 7, 2009**
*World J Gastroenterol*
2009 May 7; 15(17): 2049-2176

**Online Submissions**
wjg.wjgnet.com
www.wjgnet.com
Printed on Acid-free Paper
世界胃腸病学杂志

A Weekly Journal of Gastroenterology and Hepatology

# World Journal of Gastroenterology ®

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2007-2009

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[1]Passed away on October 20, 2007
[2]Passed away on June 11, 2007
[3]Passed away on June 14, 2008

World Journal of
Gastroenterology®
Weekly Established in October 1995
Volume 15 Number 17
May 7, 2009

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*World Journal of Gastroenterology*
**Volume** 15 **Number** 17 **May** 7, 2009

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PUBLICATION DATE
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SUBSCRIPTION
RMB 50 Yuan for each issue, RMB 2400 Yuan for one year

CSSN
ISSN 1007-9327
CN 14-1219/R





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doi:10.3748/wjg.15.2049

*World J Gastroenterol* 2009 May 7; 15(17): 2049-2066
***World Journal of Gastroenterology*** ISSN 1007-9327


*EDITORIAL*

# Intrahepatic cholestasis of pregnancy

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Author contributions: Both authors contributed equally; Geenes V and Williamson C planned the study; Geenes V wrote the first draft of the manuscript which was subsequently modified by both authors.
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Received: January 7, 2009 Revised: March 30, 2009
Accepted: April 6, 2009
Published online: May 7, 2009

## 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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Geenes V, Williamson C. Intrahepatic cholestasis of pregnancy. *World J Gastroenterol* 2009; 15(17): 2049-2066 Available from: URL: http://www.wjgnet.com/1007-9327/15/2049.asp DOI: http://dx.doi.org/10.3748/wjg.15.2049

## 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%<br>38% U | - | - | - | 7% | [12] |
| 1963-1976 | 42 (42) | 39%<br>39% S | - | - | - | 19% | [4] |
| 1965-1974 | 56 | 36%<br>36% S | 14% (NS)<br>8 B | - | 27%<br>NS | 9% | [38] |
| 1971-1972 | 116 (116) | - | 19% (labour)<br>3% LD<br>16% B or T | 7% (1)<br>3% (5) | 28%<br>7% < 37 wk<br>21% > 37 wk | - | [13] |
| 1971-1974 | 100 (100) | - | - | 10% (1)<br>8% (5) | 12%<br>NS | - | [23] |
| 1975-1984 | 83 | 44%<br>44% S | - | - | 45%<br>10% < 37 wk<br>35% > 37 wk | - | [46] |
| 1979-1981 | 18 | - | - | - | 58.30%<br>NS | 22% | [39] |
| 1980–1981 | 117 | - | 14% (antepartum)<br>4% (labour) | 10% (NS) | 16.20%<br>NS | - | [48] |
| 1988-1990 | 320 (320) | 19%<br>12% S<br>7% I | 12.8% (NS) | 8% (1)<br>2% (5) | 38%<br>13% < 37 wk<br>25% > 37 wk | - | [18] |
| 1988-1995 | 79 (79[2]) | 14%<br>14% S | 2% (NS)<br>2% B | None | 44%<br>NS | - | [57] |
| 1989-1995 | 50 | 60%<br>60% U | - | - | - | 2% | [56] |
| 1989-1997 | 206 | 27%<br>4% S<br>23% I | - | 1% (5) | 21%<br>5% < 37 wk<br>16% > 37 wk | - | [20] |
| 1990-1996 | 91 (16, 818) | 14%<br>14% U | 20% (labour) | 8% (1)<br>2% (5) | 15%<br>NS | - | [11] |
| 1999-2001 | 70 | 17%<br>6% S<br>11% I | - | - | 14%<br>4% < 37 wk<br>10% > 37 wk | 17% | [30] |
| 1999-2002 | 690 (44, 792) | 12%<br>12% U | 7%[3] | 7%[3] | 25% | - | [25] |
| 1999-2003[1] | 352 | 38%<br>16% S<br>22% I | - | - | - | - | [31] |
| 2000-2007 | 122 | 4%<br>4% S | - | 8% (1)<br>2% (5) | 13% | 7% | [49] |
| 2003-2005 | 8 | 50%<br>50% U | - | - | 62%<br>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

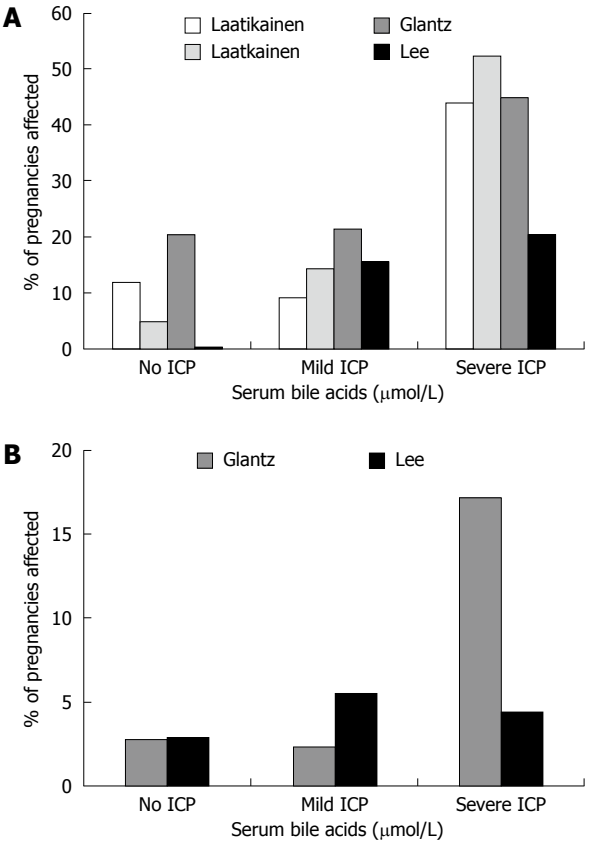


**Figure 1 Graphs showing the incidence of meconium staining of the amniotic fluid (A) and preterm labour (B) in studies of the relationship between the maternal serum bile acid level and adverse fetal outcomes[25,47-49].** The total maternal serum bile acid level was used in all studies except Laatikainen *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

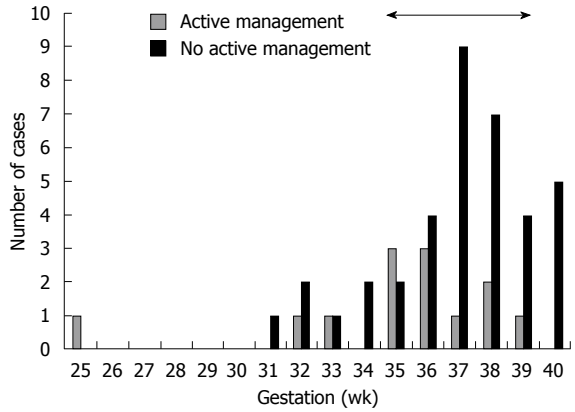


**Figure 2 Graph showing the timing of IUD associated with ICP[13,18,23,25,31,38,45,47,48,56-58].** The arrow represents six additional cases of IUD from two series with no active management reported as a range of gestational ages at the time of fetal death[45,46].

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, $P < 0.001$, $\chi^2$) (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<br>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<br>No change in any other parameter | [92] |
| 12 (12) | T3 | Serum selenium and glutathione peroxidase | ↓ Selenium in ICP<br>↓ 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<br>↑ (Double) copper in ICP<br>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.

## REFERENCES


1 **Svanborg A**. A study of recurrent jaundice in pregnancy. *Acta Obstet Gynecol Scand* 1954; **33**: 434-444
2 **Thorling L**. Jaundice in pregnancy; a clinical study. *Acta Med Scand Suppl* 1955; **302**: 1-123
3 **Fisk NM**, Bye WB, Storey GN. Maternal features of obstetric cholestasis: 20 years experience at King George V Hospital. *Aust N Z J Obstet Gynaecol* 1988; **28**: 172-176
4 **Johnston WG**, Baskett TF. Obstetric cholestasis. A 14 year review. *Am J Obstet Gynecol* 1979; **133**: 299-301
5 **Kater RM**, Mistilis SP. Obstetric cholestasis and pruritus of pregnancy. *Med J Aust* 1967; **1**: 638-640
6 **Reyes H**, Gonzalez MC, Ribalta J, Aburto H, Matus C, Schramm G, Katz R, Medina E. Prevalence of intrahepatic cholestasis of pregnancy in Chile. *Ann Intern Med* 1978; **88**: 487-493
7 **Reyes H**, Taboada G, Ribalta J. Prevalence of intrahepatic cholestasis of pregnancy in La Paz, Bolivia. *J Chronic Dis* 1979; **32**: 499-504
8 **Steel R**, Parker ML. Jaundice in pregnancy. *Med J Aust* 1973; **1**: 461
9 **Eloranta ML**, Heinonen S, Mononen T, Saarikoski S. Risk of obstetric cholestasis in sisters of index patients. *Clin Genet* 2001; **60**: 42-45
10 **Gonzalez MC**, Reyes H, Arrese M, Figueroa D, Lorca B, Andresen M, Segovia N, Molina C, Arce S. Intrahepatic cholestasis of pregnancy in twin pregnancies. *J Hepatol* 1989; **9**: 84-90
11 **Heinonen S**, Kirkinen P. Pregnancy outcome with intrahepatic cholestasis. *Obstet Gynecol* 1999; **94**: 189-193
12 **Jiang ZH**, Qiu ZD, Liu WW, Liu YH, Wang QN, Miao HZ, Zhou ZC, Wu XL, Xu BY, Gu CH. Intrahepatic cholestasis of pregnancy and its complications. Analysis of 100 cases in Chongqing area. *Chin Med J* (Engl) 1986; **99**: 957-960
13 **Laatikainen T**, Ikonen E. Fetal prognosis in obstetric hepatosis. *Ann Chir Gynaecol Fenn* 1975; **64**: 155-164
14 **Lo TK**, Lau WL, Lam HS, Leung WC, Chin RK. Obstetric cholestasis in Hong Kong--local experience with eight consecutive cases. *Hong Kong Med J* 2007; **13**: 387-391
15 **Paternoster DM**, Fabris F, Palù G, Santarossa C, Bracciante R, Snijders D, Floreani A. Intra-hepatic cholestasis of pregnancy in hepatitis C virus infection. *Acta Obstet Gynecol Scand* 2002; **81**: 99-103
16 **Perreau P**, Rouchy R. [Recurrent cholostatic jaundice of pregnancy.] *Gynecol Obstet* (Paris) 1961; **60**: 161-179
17 **Rathi U**, Bapat M, Rathi P, Abraham P. Effect of liver disease on maternal and fetal outcome--a prospective study. *Indian J Gastroenterol* 2007; **26**: 59-63
18 **Rioseco AJ**, Ivankovic MB, Manzur A, Hamed F, Kato SR, Parer JT, Germain AM. Intrahepatic cholestasis of pregnancy: a retrospective case-control study of perinatal outcome. *Am J Obstet Gynecol* 1994; **170**: 890-895
19 **Roger D**, Vaillant L, Fignon A, Pierre F, Bacq Y, Brechot JF, Grangeponte MC, Lorette G. Specific pruritic diseases of pregnancy. A prospective study of 3192 pregnant women. *Arch Dermatol* 1994; **130**: 734-739
20 **Roncaglia N**, Arreghini A, Locatelli A, Bellini P, Andreotti C, Ghidini A. Obstetric cholestasis: outcome with active

management. *Eur J Obstet Gynecol Reprod Biol* 2002; **100**: 167-170
21 **Wójcicka-Jagodzińska J**, Kuczyńska-Sicińska J, Czajkowski K, Smolarczyk R. Carbohydrate metabolism in the course of intrahepatic cholestasis in pregnancy. *Am J Obstet Gynecol* 1989; **161**: 959-964
22 **Abedin P**, Weaver JB, Egginton E. Intrahepatic cholestasis of pregnancy: prevalence and ethnic distribution. *Ethn Health* 1999; **4**: 35-37
23 **Berg B**, Helm G, Petersohn L, Tryding N. Cholestasis of pregnancy. Clinical and laboratory studies. *Acta Obstet Gynecol Scand* 1986; **65**: 107-113
24 **Brites D**, Rodrigues CM, van-Zeller H, Brito A, Silva R. Relevance of serum bile acid profile in the diagnosis of intrahepatic cholestasis of pregnancy in an high incidence area: Portugal. *Eur J Obstet Gynecol Reprod Biol* 1998; **80**: 31-38
25 **Glantz A**, Marschall HU, Mattsson LA. Intrahepatic cholestasis of pregnancy: Relationships between bile acid levels and fetal complication rates. *Hepatology* 2004; **40**: 467-474
26 **Laifer SA**, Stiller RJ, Siddiqui DS, Dunston-Boone G, Whetham JC. Ursodeoxycholic acid for the treatment of intrahepatic cholestasis of pregnancy. *J Matern Fetal Med* 2001; **10**: 131-135
27 **Lee RH**, Goodwin TM, Greenspoon J, Incerpi M. The prevalence of intrahepatic cholestasis of pregnancy in a primarily Latina Los Angeles population. *J Perinatol* 2006; **26**: 527-532
28 **Reyes H**. Sex hormones and bile acids in intrahepatic cholestasis of pregnancy. *Hepatology* 2008; **47**: 376-379
29 **Koivurova S**, Hartikainen AL, Karinen L, Gissler M, Hemminki E, Martikainen H, Tuomivaara L, Järvelin MR. The course of pregnancy and delivery and the use of maternal healthcare services after standard IVF in Northern Finland 1990-1995. *Hum Reprod* 2002; **17**: 2897-2903
30 **Kenyon AP**, Piercy CN, Girling J, Williamson C, Tribe RM, Shennan AH. Obstetric cholestasis, outcome with active management: a series of 70 cases. *BJOG* 2002; **109**: 282-288
31 **Williamson C**, Hems LM, Goulis DG, Walker I, Chambers J, Donaldson O, Swiet M, Johnston DG. Clinical outcome in a series of cases of obstetric cholestasis identified via a patient support group. *BJOG* 2004; **111**: 676-681
32 **Locatelli A**, Roncaglia N, Arreghini A, Bellini P, Vergani P, Ghidini A. Hepatitis C virus infection is associated with a higher incidence of cholestasis of pregnancy. *Br J Obstet Gynaecol* 1999; **106**: 498-500
33 **Johnson P**, Samsioe G, Gustafson A. Studies in cholestasis of pregnancy. I. Clinical aspects and liver function tests. *Acta Obstet Gynecol Scand* 1975; **54**: 77-84
34 **Reyes H**. The spectrum of liver and gastrointestinal disease seen in cholestasis of pregnancy. *Gastroenterol Clin North Am* 1992; **21**: 905-921
35 **Kenyon AP**, Piercy CN, Girling J, Williamson C, Tribe RM, Shennan AH. Pruritus may precede abnormal liver function tests in pregnant women with obstetric cholestasis: a longitudinal analysis. *BJOG* 2001; **108**: 1190-1192
36 **Lunzer MR**. Jaundice in pregnancy. *Baillieres Clin Gastroenterol* 1989; **3**: 467-483
37 **Reyes H**, Radrigan ME, Gonzalez MC, Latorre R, Ribalta J, Segovia N, Alvarez C, Andresen M, Figueroa D, Lorca B. Steatorrhea in patients with intrahepatic cholestasis of pregnancy. *Gastroenterology* 1987; **93**: 584-590
38 **Reid R**, Ivey KJ, Rencoret RH, Storey B. Fetal complications of obstetric cholestasis. *Br Med J* 1976; **1**: 870-872
39 **Shaw D**, Frohlich J, Wittmann BA, Willms M. A prospective study of 18 patients with cholestasis of pregnancy. *Am J Obstet Gynecol* 1982; **142**: 621-625
40 **Atabey S**, Duvan CI, Eren U, Turhan NO. Intrahepatic cholestasis and eclampsia: a case report. *Hypertens Pregnancy* 2007; **26**: 363-369
41 **Goulis DG**, Walker IA, de Swiet M, Redman CW, Williamson C. Preeclampsia with abnormal liver function tests is associated with cholestasis in a subgroup of cases. *Hypertens Pregnancy* 2004; **23**: 19-27
42 **Vanjak D**, Moreau R, Roche-Sicot J, Soulier A, Sicot C. Intrahepatic cholestasis of pregnancy and acute fatty liver of pregnancy. An unusual but favorable association? *Gastroenterology* 1991; **100**: 1123-1125
43 **Aytaç S**, Kargili A, Türkay C. A prolonged gestational intrahepatic cholestasis: a case report. *Turk J Gastroenterol* 2006; **17**: 206-208
44 **Olsson R**, Tysk C, Aldenborg F, Holm B. Prolonged postpartum course of intrahepatic cholestasis of pregnancy. *Gastroenterology* 1993; **105**: 267-271
45 **Davies MH**, da Silva RC, Jones SR, Weaver JB, Elias E. Fetal mortality associated with cholestasis of pregnancy and the potential benefit of therapy with ursodeoxycholic acid. *Gut* 1995; **37**: 580-584
46 **Fisk NM**, Storey GN. Fetal outcome in obstetric cholestasis. *Br J Obstet Gynaecol* 1988; **95**: 1137-1143
47 **Laatikainen T**, Ikonen E. Serum bile acids in cholestasis of pregnancy. *Obstet Gynecol* 1977; **50**: 313-318
48 **Laatikainen T**, Tulenheimo A. Maternal serum bile acid levels and fetal distress in cholestasis of pregnancy. *Int J Gynaecol Obstet* 1984; **22**: 91-94
49 **Lee RH**, Kwok KM, Ingles S, Wilson ML, Mullin P, Incerpi M, Pathak B, Goodwin TM. Pregnancy outcomes during an era of aggressive management for intrahepatic cholestasis of pregnancy. *Am J Perinatol* 2008; **25**: 341-345
50 **Ammälä P**, Kariniemi V. Short-term variability of fetal heart rate in cholestasis of pregnancy. *Am J Obstet Gynecol* 1981; **141**: 217-220
51 **Al Inizi S**, Gupta R, Gale A. Fetal tachyarrhythmia with atrial flutter in obstetric cholestasis. *Int J Gynaecol Obstet* 2006; **93**: 53-54
52 **Morrison JJ**, Rennie JM, Milton PJ. Neonatal respiratory morbidity and mode of delivery at term: influence of timing of elective caesarean section. *Br J Obstet Gynaecol* 1995; **102**: 101-106
53 **Zecca E**, Costa S, Lauriola V, Vento G, Papacci P, Romagnoli C. Bile acid pneumonia: a "new" form of neonatal respiratory distress syndrome? *Pediatrics* 2004; **114**: 269-272
54 **Zecca E**, De Luca D, Baroni S, Vento G, Tiberi E, Romagnoli C. Bile acid-induced lung injury in newborn infants: a bronchoalveolar lavage fluid study. *Pediatrics* 2008; **121**: e146-e149
55 **Zecca E**, De Luca D, Marras M, Caruso A, Bernardini T, Romagnoli C. Intrahepatic cholestasis of pregnancy and neonatal respiratory distress syndrome. *Pediatrics* 2006; **117**: 1669-1672
56 **Bacq Y**, Sapey T, Bréchot MC, Pierre F, Fignon A, Dubois F. Intrahepatic cholestasis of pregnancy: a French prospective study. *Hepatology* 1997; **26**: 358-364
57 **Alsulyman OM**, Ouzounian JG, Ames-Castro M, Goodwin TM. Intrahepatic cholestasis of pregnancy: perinatal outcome associated with expectant management. *Am J Obstet Gynecol* 1996; **175**: 957-960
58 **Sentilhes L**, Verspyck E, Pia P, Marpeau L. Fetal death in a patient with intrahepatic cholestasis of pregnancy. *Obstet Gynecol* 2006; **107**: 458-460
59 **Lunzer M**, Barnes P, Byth K, O'Halloran M. Serum bile acid concentrations during pregnancy and their relationship to obstetric cholestasis. *Gastroenterology* 1986; **91**: 825-829
60 **Girling JC**, Dow E, Smith JH. Liver function tests in pre-eclampsia: importance of comparison with a reference range derived for normal pregnancy. *Br J Obstet Gynaecol* 1997; **104**: 246-250
61 **Bacq Y**, Zarka O, Bréchot JF, Mariotte N, Vol S, Tichet J, Weill J. Liver function tests in normal pregnancy: a

prospective study of 103 pregnant women and 103 matched controls. *Hepatology* 1996; **23**: 1030-1034
62 **Heikkinen J**. Serum bile acids in the early diagnosis of intrahepatic cholestasis of pregnancy. *Obstet Gynecol* 1983; **61**: 581-587
63 **Milkiewicz P**, Gallagher R, Chambers J, Eggington E, Weaver J, Elias E. Obstetric cholestasis with elevated gamma glutamyl transpeptidase: incidence, presentation and treatment. *J Gastroenterol Hepatol* 2003; **18**: 1283-1286
64 **Beckett GJ**, Hayes JD. Glutathione S-transferases: biomedical applications. *Adv Clin Chem* 1993; **30**: 281-380
65 **Hayes PC**, Hussey AJ, Keating J, Bouchier IA, Williams R, Beckett GJ, Hayes JD. Glutathione S-transferase levels in autoimmune chronic active hepatitis: a more sensitive index of hepatocellular damage than aspartate transaminase. *Clin Chim Acta* 1988; **172**: 211-216
66 **Knapen MF**, Peters WH, Mulder TP, Steegers EA. A marker for hepatocellular damage. *Lancet* 2000; **355**: 1463-1464
67 **Dann AT**, Kenyon AP, Seed PT, Poston L, Shennan AH, Tribe RM. Glutathione S-transferase and liver function in intrahepatic cholestasis of pregnancy and pruritus gravidarum. *Hepatology* 2004; **40**: 1406-1414
68 **Joutsiniemi T**, Leino R, Timonen S, Pulkki K, Ekblad U. Hepatocellular enzyme glutathione S-transferase alpha and intrahepatic cholestasis of pregnancy. *Acta Obstet Gynecol Scand* 2008; **87**: 1280-1284
69 **Russell DW**. The enzymes, regulation, and genetics of bile acid synthesis. *Annu Rev Biochem* 2003; **72**: 137-174
70 **Trauner M**, Boyer JL. Bile salt transporters: molecular characterization, function, and regulation. *Physiol Rev* 2003; **83**: 633-671
71 **Carter J**. Serum bile acids in normal pregnancy. *Br J Obstet Gynaecol* 1991; **98**: 540-543
72 **Fulton IC**, Douglas JG, Hutchon DJ, Beckett GJ. Is normal pregnancy cholestatic? *Clin Chim Acta* 1983; **130**: 171-176
73 **Heikkinen J**, Mäentausta O, Ylöstalo P, Jänne O. Changes in serum bile acid concentrations during normal pregnancy, in patients with intrahepatic cholestasis of pregnancy and in pregnant women with itching. *Br J Obstet Gynaecol* 1981; **88**: 240-245
74 **Pascual MJ**, Serrano MA, El-Mir MY, Macias RI, Jiménez F, Marin JJ. Relationship between asymptomatic hypercholanaemia of pregnancy and progesterone metabolism. *Clin Sci* (Lond) 2002; **102**: 587-593
75 **Castaño G**, Lucangioli S, Sookoian S, Mesquida M, Lemberg A, Di Scala M, Franchi P, Carducci C, Tripodi V. Bile acid profiles by capillary electrophoresis in intrahepatic cholestasis of pregnancy. *Clin Sci* (Lond) 2006; **110**: 459-465
76 **Walker IA**, Nelson-Piercy C, Williamson C. Role of bile acid measurement in pregnancy. *Ann Clin Biochem* 2002; **39**: 105-113
77 **Sjövall K**, Sjövall J. Serum bile acid levels in pregnancy with pruritus (bile acids and steroids 158). *Clin Chim Acta* 1966; **13**: 207-211
78 **Laatikainen TJ**. Fetal bile acid levels in pregnancies complicated by maternal intrahepatic cholestasis. *Am J Obstet Gynecol* 1975; **122**: 852-856
79 **Colombo C**, Roda A, Roda E, Buscaglia M, dell'Agnola CA, Filippetti P, Ronchi M, Sereni F. Correlation between fetal and maternal serum bile acid concentrations. *Pediatr Res* 1985; **19**: 227-231
80 **Heikkinen J**. Effect of a standard test meal on serum bile acid levels in healthy nonpregnant and pregnant women and in patients with intrahepatic cholestasis of pregnancy. *Ann Clin Res* 1983; **15**: 183-188
81 **Laatikainen T**. Postprandial serum bile acids in cholestasis of pregnancy. *Ann Clin Res* 1978; **10**: 307-312
82 **Laatikainen T**, Lehtonen P, Hesso A. Biliary bile acids in uncomplicated pregnancy and in cholestasis of pregnancy. *Clin Chim Acta* 1978; **85**: 145-150
83 **Johnson P**. Studies in cholestasis of pregnancy with special reference to lipids and lipoproteins. *Acta Obstet Gynecol Scand Suppl* 1973; **27**: 1-80
84 **Nikkilä K**, Riikonen S, Lindfors M, Miettinen TA. Serum squalene and noncholesterol sterols before and after delivery in normal and cholestatic pregnancy. *J Lipid Res* 1996; **37**: 2687-2695
85 **Dann AT**, Kenyon AP, Wierzbicki AS, Seed PT, Shennan AH, Tribe RM. Plasma lipid profiles of women with intrahepatic cholestasis of pregnancy. *Obstet Gynecol* 2006; **107**: 106-114
86 **Dong M**, Xie X, Wang Z, He J, Zhou J, Cheng Q. Impaired mixed lymphocyte reaction in intrahepatic cholestasis of pregnancy. *Gynecol Obstet Invest* 2002; **54**: 191-195
87 **Eloranta ML**, Heinonen S, Kirkinen P. Intrahepatic cholestasis of pregnancy has no effect on maternal serum second trimester alpha-fetoprotein and hCG. *Acta Obstet Gynecol Scand* 2000; **79**: 548-552
88 **Garoff L**. Prediction of fetal outcome by urinary estriol, maternal serum placental lactogen, and alpha-fetoprotein in diabetes and hepatosis of pregnancy. *Obstet Gynecol* 1976; **48**: 659-666
89 **Heikinheimo M**, Unnérus HA, Ranta T, Jalanko H, Seppälä M. Pregnancy-specific beta-1-glycoprotein levels in cholestasis of pregnancy. *Obstet Gynecol* 1978; **52**: 276-278
90 **Kauppila A**, Korpela H, Mäkilä UM, Yrjänheikki E. Low serum selenium concentration and glutathione peroxidase activity in intrahepatic cholestasis of pregnancy. *Br Med J* (Clin Res Ed) 1987; **294**: 150-152
91 **Kiilholma P**. Serum copper and zinc concentrations in intrahepatic cholestasis of pregnancy: a controlled study. *Eur J Obstet Gynecol Reprod Biol* 1986; **21**: 207-212
92 **Kuoppala T**, Tuimala R, Parviainen M, Koskinen T. Vitamin D and mineral metabolism in intrahepatic cholestasis of pregnancy. *Eur J Obstet Gynecol Reprod Biol* 1986; **23**: 45-51
93 **Pineda G**, Aguayo J, Ribalta J, González M, Reyes H. [Thyroid function tests in normal pregnant women (third trimester) and in pregnant women with pregnancy cholestasis or with acute hepatitis] *Rev Med Chil* 2000; **128**: 35-43
94 **Ranta T**, Unnérus HA, Rossi J, Seppälä M. Elevated plasma prolactin concentration in cholestasis of pregnancy. *Am J Obstet Gynecol* 1979; **134**: 1-3
95 **Räty R**, Anttila L, Virtanen A, Koskinen P, Laitinen P, Mörsky P, Tiitinen A, Martikainen H, Ekblad U. Maternal midtrimester free beta-HCG and AFP serum levels in spontaneous singleton pregnancies complicated by gestational diabetes mellitus, pregnancy-induced hypertension or obstetric cholestasis. *Prenat Diagn* 2003; **23**: 1045-1048
96 **Reyes H**, Báez ME, González MC, Hernández I, Palma J, Ribalta J, Sandoval L, Zapata R. Selenium, zinc and copper plasma levels in intrahepatic cholestasis of pregnancy, in normal pregnancies and in healthy individuals, in Chile. *J Hepatol* 2000; **32**: 542-549
97 **Smolarczyk R**, Wójcicka-Jagodzińska J, Piekarski P, Romejko E, Czajkowski K. The biochemical functions of the renal tubules and glomeruli in the course of intrahepatic cholestasis in pregnancy. *Eur J Obstet Gynecol Reprod Biol* 2000; **89**: 35-39
98 **Tiitinen A**, Laatikainen T, Rutanen EM, Ranta T, Koistinen R, Bohn H, Seppälä M. Placental protein 10 (PP10) in normal pregnancy and cholestasis of pregnancy. *Br J Obstet Gynaecol* 1985; **92**: 1137-1140
99 **Wang Z**, Dong M, Chu H, He J. Increased serum levels of neopterin and soluble interleukin-2 receptor in intrahepatic cholestasis of pregnancy. *Acta Obstet Gynecol Scand* 2004; **83**: 1067-1070
100 **Wojcicka J**, Sienko J, Smolarczyk R, Romejko E, Grymowicz M, Czajkowski K. Alpha-hydroxybutyrate dehydrogenase

activity in intrahepatic cholestasis of pregnancy. *Int J Gynaecol Obstet* 2005; **89**: 247-250
101 **Thomassen PA**. Urinary bile acids in late pregnancy and in recurrent cholestasis of pregnancy. *Eur J Clin Invest* 1979; **9**: 425-432
102 **Thomassen PA**. Urinary bile acids during development of recurrent cholestasis of pregnancy. *Eur J Clin Invest* 1979; **9**: 417-423
103 **Ko CW**, Beresford SA, Schulte SJ, Matsumoto AM, Lee SP. Incidence, natural history, and risk factors for biliary sludge and stones during pregnancy. *Hepatology* 2005; **41**: 359-365
104 **Tsimoyiannis EC**, Antoniou NC, Tsaboulas C, Papanikolaou N. Cholelithiasis during pregnancy and lactation. Prospective study. *Eur J Surg* 1994; **160**: 627-631
105 **Kirkinen P**, Ylöstalo P, Heikkinen J, Mäentausta O. Gallbladder function and maternal bile acids in intrahepatic cholestasis of pregnancy. *Eur J Obstet Gynecol Reprod Biol* 1984; **18**: 29-34
106 **Ylöstalo P**, Kirkinen P, Heikkinen J, Mäentausta O, Järvinen PA. Gall bladder volume and serum bile acids in cholestasis of pregnancy. *Br J Obstet Gynaecol* 1982; **89**: 59-61
107 **Ylötalo P**, Kirkinen P, Heikkinen J, Mäentausta O. Gallbladder volume in cholestasis of pregnancy. *N Engl J Med* 1981; **304**: 359
108 **Ikonen E**. Jaundice in Late Pregnancy. *Acta Obstet Gynecol Scand* 1964; **43** Suppl 5: 1-130
109 **Eliakim M**, Sadovsky E, Stein O, Shenkar YG. Recurrent cholestatic jaundice of pregnancy. Report of five cases and electron microscopic observations. *Arch Intern Med* 1966; **117**: 696-705
110 **Reyes H**, Ribalta J, González-Cerón M. Idiopathic cholestasis of pregnancy in a large kindred. *Gut* 1976; **17**: 709-713
111 **Dixon PH**, Williamson C. The molecular genetics of intrahepatic cholestasis of pregnancy. *Obstet Med* 2008; **1**: 65-71
112 **Ruetz S**, Gros P. Phosphatidylcholine translocase: a physiological role for the mdr2 gene. *Cell* 1994; **77**: 1071-1081
113 **Smith AJ**, Timmermans-Hereijgers JL, Roelofsen B, Wirtz KW, van Blitterswijk WJ, Smit JJ, Schinkel AH, Borst P. The human MDR3 P-glycoprotein promotes translocation of phosphatidylcholine through the plasma membrane of fibroblasts from transgenic mice. *FEBS Lett* 1994; **354**: 263-266
114 **van Helvoort A**, Smith AJ, Sprong H, Fritzsche I, Schinkel AH, Borst P, van Meer G. MDR1 P-glycoprotein is a lipid translocase of broad specificity, while MDR3 P-glycoprotein specifically translocates phosphatidylcholine. *Cell* 1996; **87**: 507-517
115 **de Vree JM**, Jacquemin E, Sturm E, Cresteil D, Bosma PJ, Aten J, Deleuze JF, Desrochers M, Burdelski M, Bernard O, Oude Elferink RP, Hadchouel M. Mutations in the MDR3 gene cause progressive familial intrahepatic cholestasis. *Proc Natl Acad Sci USA* 1998; **95**: 282-287
116 **Rosmorduc O**, Hermelin B, Poupon R. MDR3 gene defect in adults with symptomatic intrahepatic and gallbladder cholesterol cholelithiasis. *Gastroenterology* 2001; **120**: 1459-1467
117 **Jacquemin E**, Cresteil D, Manouvrier S, Boute O, Hadchouel M. Heterozygous non-sense mutation of the MDR3 gene in familial intrahepatic cholestasis of pregnancy. *Lancet* 1999; **353**: 210-211
118 **Eloranta ML**, Heiskanen JT, Hiltunen MJ, Mannermaa AJ, Punnonen KR, Heinonen ST. Multidrug resistance 3 gene mutation 1712delT and estrogen receptor alpha gene polymorphisms in Finnish women with obstetric cholestasis. *Eur J Obstet Gynecol Reprod Biol* 2002; **105**: 132-135
119 **Dixon PH**, Weerasekera N, Linton KJ, Donaldson O, Chambers J, Egginton E, Weaver J, Nelson-Piercy C, de Swiet M, Warnes G, Elias E, Higgins CF, Johnston DG, McCarthy MI, Williamson C. Heterozygous MDR3 missense mutation associated with intrahepatic cholestasis of pregnancy: evidence for a defect in protein trafficking. *Hum Mol Genet* 2000; **9**: 1209-1217
120 **Floreani A**, Carderi I, Paternoster D, Soardo G, Azzaroli F, Esposito W, Montagnani M, Marchesoni D, Variola A, Rosa Rizzotto E, Braghin C, Mazzella G. Hepatobiliary phospholipid transporter ABCB4, MDR3 gene variants in a large cohort of Italian women with intrahepatic cholestasis of pregnancy. *Dig Liver Dis* 2008; **40**: 366-370
121 **Floreani A**, Carderi I, Paternoster D, Soardo G, Azzaroli F, Esposito W, Variola A, Tommasi AM, Marchesoni D, Braghin C, Mazzella G. Intrahepatic cholestasis of pregnancy: three novel MDR3 gene mutations. *Aliment Pharmacol Ther* 2006; **23**: 1649-1653
122 **Gendrot C**, Bacq Y, Brechot MC, Lansac J, Andres C. A second heterozygous MDR3 nonsense mutation associated with intrahepatic cholestasis of pregnancy. *J Med Genet* 2003; **40**: e32
123 **Keitel V**, Vogt C, Häussinger D, Kubitz R. Combined mutations of canalicular transporter proteins cause severe intrahepatic cholestasis of pregnancy. *Gastroenterology* 2006; **131**: 624-629
124 **Lucena JF**, Herrero JI, Quiroga J, Sangro B, Garcia-Foncillas J, Zabalegui N, Sola J, Herraiz M, Medina JF, Prieto J. A multidrug resistance 3 gene mutation causing cholelithiasis, cholestasis of pregnancy, and adulthood biliary cirrhosis. *Gastroenterology* 2003; **124**: 1037-1042
125 **Müllenbach R**, Linton KJ, Wiltshire S, Weerasekera N, Chambers J, Elias E, Higgins CF, Johnston DG, McCarthy MI, Williamson C. ABCB4 gene sequence variation in women with intrahepatic cholestasis of pregnancy. *J Med Genet* 2003; **40**: e70
126 **Pauli-Magnus C**, Lang T, Meier Y, Zodan-Marin T, Jung D, Breymann C, Zimmermann R, Kenngott S, Beuers U, Reichel C, Kerb R, Penger A, Meier PJ, Kullak-Ublick GA. Sequence analysis of bile salt export pump (ABCB11) and multidrug resistance p-glycoprotein 3 (ABCB4, MDR3) in patients with intrahepatic cholestasis of pregnancy. *Pharmacogenetics* 2004; **14**: 91-102
127 **Wasmuth HE**, Glantz A, Keppeler H, Simon E, Bartz C, Rath W, Mattsson LA, Marschall HU, Lammert F. Intrahepatic cholestasis of pregnancy: the severe form is associated with common variants of the hepatobiliary phospholipid transporter ABCB4 gene. *Gut* 2007; **56**: 265-270
128 **Müllenbach R**, Bennett A, Tetlow N, Patel N, Hamilton G, Cheng F, Chambers J, Howard R, Taylor-Robinson SD, Williamson C. ATP8B1 mutations in British cases with intrahepatic cholestasis of pregnancy. *Gut* 2005; **54**: 829-834
129 **Painter JN**, Savander M, Ropponen A, Nupponen N, Riikonen S, Ylikorkala O, Lehesjoki AE, Aittomäki K. Sequence variation in the ATP8B1 gene and intrahepatic cholestasis of pregnancy. *Eur J Hum Genet* 2005; **13**: 435-439
130 **Eloranta ML**, Häkli T, Hiltunen M, Helisalmi S, Punnonen K, Heinonen S. Association of single nucleotide polymorphisms of the bile salt export pump gene with intrahepatic cholestasis of pregnancy. *Scand J Gastroenterol* 2003; **38**: 648-652
131 **Painter JN**, Savander M, Sistonen P, Lehesjoki AE, Aittomäki K. A known polymorphism in the bile salt export pump gene is not a risk allele for intrahepatic cholestasis of pregnancy. *Scand J Gastroenterol* 2004; **39**: 694-695
132 **Savander M**, Ropponen A, Avela K, Weerasekera N, Cormand B, Hirvioja ML, Riikonen S, Ylikorkala O, Lehesjoki AE, Williamson C, Aittomäki K. Genetic evidence of heterogeneity in intrahepatic cholestasis of pregnancy. *Gut* 2003; **52**: 1025-1029
133 **Meier Y**, Zodan T, Lang C, Zimmermann R, Kullak-Ublick GA, Meier PJ, Stieger B, Pauli-Magnus C. Increased susceptibility for intrahepatic cholestasis of pregnancy

and contraceptive-induced cholestasis in carriers of the 1331T>C polymorphism in the bile salt export pump. *World J Gastroenterol* 2008; **14**: 38-45

134 **Dixon PH**, van Mil SW, Chambers J, Strautnieks S, Thompson RJ, Lammert F, Kubitz R, Keitel V, Glantz A, Mattsson LA, Marschall HU, Molokhia M, Moore GE, Linton KJ, Williamson C. Contribution of variant alleles of ABCB11 to susceptibility to intrahepatic cholestasis of pregnancy. *Gut* 2009; **58**: 537-544

135 **Sookoian S**, Castaño G, Burgueño A, Gianotti TF, Pirola CJ. Association of the multidrug-resistance-associated protein gene (ABCC2) variants with intrahepatic cholestasis of pregnancy. *J Hepatol* 2008; **48**: 125-132

136 **Van Mil SW**, Milona A, Dixon PH, Mullenbach R, Geenes VL, Chambers J, Shevchuk V, Moore GE, Lammert F, Glantz AG, Mattsson LA, Whittaker J, Parker MG, White R, Williamson C. Functional variants of the central bile acid sensor FXR identified in intrahepatic cholestasis of pregnancy. *Gastroenterology* 2007; **133**: 507-516

137 **Reyes H**, Ribalta J, González MC, Segovia N, Oberhauser E. Sulfobromophthalein clearance tests before and after ethinyl estradiol administration, in women and men with familial history of intrahepatic cholestasis of pregnancy. *Gastroenterology* 1981; **81**: 226-231

138 **Barth A**, Klinger G, Rost M. Influence of ethinyloestradiol propanolsulphonate on serum bile acids in healthy volunteers. *Exp Toxicol Pathol* 2003; **54**: 381-386

139 **Stieger B**, Fattinger K, Madon J, Kullak-Ublick GA, Meier PJ. Drug- and estrogen-induced cholestasis through inhibition of the hepatocellular bile salt export pump (Bsep) of rat liver. *Gastroenterology* 2000; **118**: 422-430

140 **Crocenzi FA**, Mottino AD, Cao J, Veggi LM, Pozzi EJ, Vore M, Coleman R, Roma MG. Estradiol-17beta-D-glucuronide induces endocytic internalization of Bsep in rats. *Am J Physiol Gastrointest Liver Physiol* 2003; **285**: G449-G459

141 **Simon FR**, Fortune J, Iwahashi M, Qadri I, Sutherland E. Multihormonal regulation of hepatic sinusoidal Ntcp gene expression. *Am J Physiol Gastrointest Liver Physiol* 2004; **287**: G782-G794

142 **Yamamoto Y**, Moore R, Hess HA, Guo GL, Gonzalez FJ, Korach KS, Maronpot RR, Negishi M. Estrogen receptor alpha mediates 17alpha-ethynylestradiol causing hepatotoxicity. *J Biol Chem* 2006; **281**: 16625-16631

143 **Geier A**, Dietrich CG, Gerloff T, Haendly J, Kullak-Ublick GA, Stieger B, Meier PJ, Matern S, Gartung C. Regulation of basolateral organic anion transporters in ethinylestradiol-induced cholestasis in the rat. *Biochim Biophys Acta* 2003; **1609**: 87-94

144 **Bacq Y**, Myara A, Brechot MC, Hamon C, Studer E, Trivin F, Metman EH. Serum conjugated bile acid profile during intrahepatic cholestasis of pregnancy. *J Hepatol* 1995; **22**: 66-70

145 **Benifla JL**, Dumont M, Levardon M, Foucher E, Cadiot G, Crenn-Hebert C, Heid M, Lelaidier C, Rosenbaum A, Bernuau J, Erlinger S, Frydman R, Madelenat P. [Effects of micronized natural progesterone on the liver during the third trimester of pregnancy] *Contracept Fertil Sex* 1997; **25**: 165-169

146 **Meng LJ**, Reyes H, Axelson M, Palma J, Hernandez I, Ribalta J, Sjövall J. Progesterone metabolites and bile acids in serum of patients with intrahepatic cholestasis of pregnancy: effect of ursodeoxycholic acid therapy. *Hepatology* 1997; **26**: 1573-1579

147 **Meng LJ**, Reyes H, Palma J, Hernandez I, Ribalta J, Sjövall J. Profiles of bile acids and progesterone metabolites in the urine and serum of women with intrahepatic cholestasis of pregnancy. *J Hepatol* 1997; **27**: 346-357

148 **Laatikainen TJ**, Peltonen JI, Nylander PL. Effect of maternal intrahepatic cholestasis on fetal steroid metabolism. *J Clin Invest* 1974; **53**: 1709-1715

149 **Vallejo M**, Briz O, Serrano MA, Monte MJ, Marin JJ. Potential role of trans-inhibition of the bile salt export pump by progesterone metabolites in the etiopathogenesis of intrahepatic cholestasis of pregnancy. *J Hepatol* 2006; **44**: 1150-1157

150 **Lang C**, Meier Y, Stieger B, Beuers U, Lang T, Kerb R, Kullak-Ublick GA, Meier PJ, Pauli-Magnus C. Mutations and polymorphisms in the bile salt export pump and the multidrug resistance protein 3 associated with drug-induced liver injury. *Pharmacogenet Genomics* 2007; **17**: 47-60

151 **Reyes H**, Zapata R, Hernández I, Gotteland M, Sandoval L, Jirón MI, Palma J, Almuna R, Silva JJ. Is a leaky gut involved in the pathogenesis of intrahepatic cholestasis of pregnancy? *Hepatology* 2006; **43**: 715-722

152 **Kirby J**, Heaton KW, Burton JL. Pruritic effect of bile salts. *Br Med J* 1974; **4**: 693-695

153 **Varadi DP**. Pruritus induced by crude bile and purified bile acids. Experimental production of pruritus in human skin. *Arch Dermatol* 1974; **109**: 678-681

154 **Ghent CN**, Bloomer JR, Klatskin G. Elevations in skin tissue levels of bile acids in human cholestasis: relation to serum levels and topruritus. *Gastroenterology* 1977; **73**: 1125-1130

155 **Glantz A**, Reilly SJ, Benthin L, Lammert F, Mattsson LA, Marschall HU. Intrahepatic cholestasis of pregnancy: Amelioration of pruritus by UDCA is associated with decreased progesterone disulphates in urine. *Hepatology* 2008; **47**: 544-551

156 **Schumann R**, Hudcova J. Cholestasis of pregnancy, pruritus and 5-hydroxytryptamine 3 receptor antagonists. *Acta Obstet Gynecol Scand* 2004; **83**: 861-862

157 **Schwörer H**, Ramadori G. Improvement of cholestatic pruritus by ondansetron. *Lancet* 1993; **341**: 1277

158 **Marin JJ**, Serrano MA, el-Mir MY, Eleno N, Boyd CA. Bile acid transport by basal membrane vesicles of human term placental trophoblast. *Gastroenterology* 1990; **99**: 1431-1438

159 **Serrano MA**, Brites D, Larena MG, Monte MJ, Bravo MP, Oliveira N, Marin JJ. Beneficial effect of ursodeoxycholic acid on alterations induced by cholestasis of pregnancy in bile acid transport across the human placenta. *J Hepatol* 1998; **28**: 829-839

160 **Campos GA**, Guerra FA, Israel EJ. Effects of cholic acid infusion in fetal lambs. *Acta Obstet Gynecol Scand* 1986; **65**: 23-26

161 **Falconer JD**, Smith AN, Eastwood MA. The effects of bile acids on colonic motility in the rabbit. *Q J Exp Physiol Cogn Med Sci* 1980; **65**: 135-144

162 **Kirwan WO**, Smith AN, Mitchell WD, Falconer JD, Eastwood MA. Bile acids and colonic motility in the rabbit and the human. *Gut* 1975; **16**: 894-902

163 **Williamson C**, Gorelik J, Eaton BM, Lab M, de Swiet M, Korchev Y. The bile acid taurocholate impairs rat cardiomyocyte function: a proposed mechanism for intrauterine fetal death in obstetric cholestasis. *Clin Sci* (Lond) 2001; **100**: 363-369

164 **Campos GA**, Castillo RJ, Toro FG. [Effect of bile acids on the myometral contractility of the isolated pregnant uterus] *Rev Chil Obstet Ginecol* 1988; **53**: 229-233

165 **Germain AM**, Kato S, Carvajal JA, Valenzuela GJ, Valdes GL, Glasinovic JC. Bile acids increase response and expression of human myometrial oxytocin receptor. *Am J Obstet Gynecol* 2003; **189**: 577-582

166 **Israel EJ**, Guzman ML, Campos GA. Maximal response to oxytocin of the isolated myometrium from pregnant patients with intrahepatic cholestasis. *Acta Obstet Gynecol Scand* 1986; **65**: 581-582

167 **Brown ES**. Aspiration and lung surfactant. *Anesth Analg* 1967; **46**: 665-672

168 **Porembka DT**, Kier A, Sehlhorst S, Boyce S, Orlowski JP, Davis K Jr. The pathophysiologic changes following bile aspiration in a porcine lung model. *Chest* 1993; **104**: 919-924

169 **Kaneko T**, Sato T, Katsuya H, Miyauchi Y. Surfactant therapy for pulmonary edema due to intratracheally injected bile acid. *Crit Care Med* 1990; **18**: 77-83

170 **Costoya AL**, Leontic EA, Rosenberg HG, Delgado MA. Morphological study of placental terminal villi in intrahepatic cholestasis of pregnancy: histochemistry, light and electron microscopy. *Placenta* 1980; **1**: 361-368

171 **Liebhart M**, Wójcicka J. Microscopic patterns of placenta in cases of pregnancy complicated by intrahepatic cholestasis (idiopathic jaundice). *Pol Med J* 1970; **9**: 1589-1600

172 **Perez MJ**, Macias RI, Marin JJ. Maternal cholestasis induces placental oxidative stress and apoptosis. Protective effect of ursodeoxycholic acid. *Placenta* 2006; **27**: 34-41

173 **Miller PW**, Coen RW, Benirschke K. Dating the time interval from meconium passage to birth. *Obstet Gynecol* 1985; **66**: 459-462

174 **Altshuler G**, Hyde S. Meconium-induced vasocontraction: a potential cause of cerebral and other fetal hypoperfusion and of poor pregnancy outcome. *J Child Neurol* 1989; **4**: 137-142

175 **Rodrigues CM**, Marín JJ, Brites D. Bile acid patterns in meconium are influenced by cholestasis of pregnancy and not altered by ursodeoxycholic acid treatment. *Gut* 1999; **45**: 446-452

176 **Sepúlveda WH**, González C, Cruz MA, Rudolph MI. Vasoconstrictive effect of bile acids on isolated human placental chorionic veins. *Eur J Obstet Gynecol Reprod Biol* 1991; **42**: 211-215

177 **Matos A**, Bernardes J, Ayres-de-Campos D, Patrício B. Antepartum fetal cerebral hemorrhage not predicted by current surveillance methods in cholestasis of pregnancy. *Obstet Gynecol* 1997; **89**: 803-804

178 **Medina Lomelí JM**, Medina Castro N. [Intrahepatic cholestasis of pregnancy, an unpredictable fetal risk: report of a case and review of the literature] *Ginecol Obstet Mex* 2000; **68**: 486-488

179 **Lazaridis KN**, Gores GJ, Lindor KD. Ursodeoxycholic acid 'mechanisms of action and clinical use in hepatobiliary disorders'. *J Hepatol* 2001; **35**: 134-146

180 **Rodrigues CM**, Fan G, Wong PY, Kren BT, Steer CJ. Ursodeoxycholic acid may inhibit deoxycholic acid-induced apoptosis by modulating mitochondrial transmembrane potential and reactive oxygen species production. *Mol Med* 1998; **4**: 165-178

181 **Palma J**, Reyes H, Ribalta J, Iglesias J, Gonzalez MC, Hernandez I, Alvarez C, Molina C, Danitz AM. Effects of ursodeoxycholic acid in patients with intrahepatic cholestasis of pregnancy. *Hepatology* 1992; **15**: 1043-1047

182 **Diaferia A**, Nicastri PL, Tartagni M, Loizzi P, Iacovizzi C, Di Leo A. Ursodeoxycholic acid therapy in pregnant women with cholestasis. *Int J Gynaecol Obstet* 1996; **52**: 133-140

183 **Nicastri PL**, Diaferia A, Tartagni M, Loizzi P, Fanelli M. A randomised placebo-controlled trial of ursodeoxycholic acid and S-adenosylmethionine in the treatment of intrahepatic cholestasis of pregnancy. *Br J Obstet Gynaecol* 1998; **105**: 1205-1207

184 **Palma J**, Reyes H, Ribalta J, Hernández I, Sandoval L, Almuna R, Liepins J, Lira F, Sedano M, Silva O, Tohá D, Silva JJ. Ursodeoxycholic acid in the treatment of cholestasis of pregnancy: a randomized, double-blind study controlled with placebo. *J Hepatol* 1997; **27**: 1022-1028

185 **Berkane N**, Cocheton JJ, Brehier D, Merviel P, Wolf C, Lefèvre G, Uzan S. Ursodeoxycholic acid in intrahepatic cholestasis of pregnancy. A retrospective study of 19 cases. *Acta Obstet Gynecol Scand* 2000; **79**: 941-946

186 **Floreani A**, Paternoster D, Grella V, Sacco S, Gangemi M, Chiaramonte M. Ursodeoxycholic acid in intrahepatic cholestasis of pregnancy. *Br J Obstet Gynaecol* 1994; **101**: 64-65

187 **Mazzella G**, Rizzo N, Azzaroli F, Simoni P, Bovicelli L, Miracolo A, Simonazzi G, Colecchia A, Nigro G, Mwangemi C, Festi D, Roda E. Ursodeoxycholic acid administration in patients with cholestasis of pregnancy: effects on primary bile acids in babies and mothers. *Hepatology* 2001; **33**: 504-508

188 **Zapata R**, Sandoval L, Palma J, Hernández I, Ribalta J, Reyes H, Sedano M, Tohá D, Silva JJ. Ursodeoxycholic acid in the treatment of intrahepatic cholestasis of pregnancy. A 12-year experience. *Liver Int* 2005; **25**: 548-554

189 **Glantz A**, Marschall HU, Lammert F, Mattsson LA. Intrahepatic cholestasis of pregnancy: a randomized controlled trial comparing dexamethasone and ursodeoxycholic acid. *Hepatology* 2005; **42**: 1399-1405

190 **Brites D**, Rodrigues CM, Oliveira N, Cardoso M, Graça LM. Correction of maternal serum bile acid profile during ursodeoxycholic acid therapy in cholestasis of pregnancy. *J Hepatol* 1998; **28**: 91-98

191 **Brites D**, El-Mir MY, Oliveira N, Marín JJG. Amniotic fluid bile acid changes in the course of ursodeoxycholic acid therapy in intrahepatic cholestasis of pregnancy [Abstract]. *J Hepatol* 1997; **26**: 164A

192 **Brites D**, El-Mir MY, Rodrigues CMP, van-Zeller H, Marín JJG. Bile acid composition of amniotic fluid and maternal serum in cholestasis of pregnancy and effect of ursodeoxycholic acid [Abstract]. *J Hepatol* 1998; **28**: 125A

193 **Brites D**, Rodrigues CM. Elevated levels of bile acids in colostrum of patients with cholestasis of pregnancy are decreased following ursodeoxycholic acid therapy [see comemnts] *J Hepatol* 1998; **29**: 743-751

194 **Azzaroli F**, Mennone A, Feletti V, Simoni P, Baglivo E, Montagnani M, Rizzo N, Pelusi G, DE Aloysio D, Lodato F, Festi D, Colecchia A, Roda E, Boyer JL, Mazzella G. Clinical trial: modulation of human placental multidrug resistance proteins in cholestasis of pregnancy by ursodeoxycholic acid. *Aliment Pharmacol Ther* 2007; **26**: 1139-1146

195 **Serrano MA**, Macias RI, Vallejo M, Briz O, Bravo A, Pascual MJ, St-Pierre MV, Stieger B, Meier PJ, Marin JJ. Effect of ursodeoxycholic acid on the impairment induced by maternal cholestasis in the rat placenta-maternal liver tandem excretory pathway. *J Pharmacol Exp Ther* 2003; **305**: 515-524

196 **Gorelik J**, Shevchuk AI, Diakonov I, de Swiet M, Lab M, Korchev Y, Williamson C. Dexamethasone and ursodeoxycholic acid protect against the arrhythmogenic effect of taurocholate in an in vitro study of rat cardiomyocytes. *BJOG* 2003; **110**: 467-474

197 **Kauppila A**, Jouppila P, Karvonen P, Tuimala R, Ylikorkala O. Effect of dexamethasone on blood levels of ACTH, cortisol, progesterone, estradiol and estriol during late pregnancy. *Int J Gynaecol Obstet* 1976; **14**: 177-181

198 **Kauppila A**, Tuimala R, Ylikorkala O, Reinilä M, Ylöstalo P. Placental steroid synthesis from DHEAS during dexamethasone therapy. *Obstet Gynecol* 1979; **54**: 39-42

199 **Hirvioja ML**, Tuimala R, Vuori J. The treatment of intrahepatic cholestasis of pregnancy by dexamethasone. *Br J Obstet Gynaecol* 1992; **99**: 109-111

200 **Diac M**, Kenyon A, Nelson-Piercy C, Girling J, Cheng F, Tribe RM, Goodman J, Shennan A, Williamson C. Dexamethasone in the treatment of obstetric cholestasis: a case series. *J Obstet Gynaecol* 2006; **26**: 110-114

201 **Kretowicz E**, McIntyre HD. Intrahepatic cholestasis of pregnancy, worsening after dexamethasone. *Aust N Z J Obstet Gynaecol* 1994; **34**: 211-213

202 **Bloom SL**, Sheffield JS, McIntire DD, Leveno KJ. Antenatal dexamethasone and decreased birth weight. *Obstet Gynecol* 2001; **97**: 485-490

203 **Modi N**, Lewis H, Al-Naqeeb N, Ajayi-Obe M, Doré CJ, Rutherford M. The effects of repeated antenatal glucocorticoid therapy on the developing brain. *Pediatr Res* 2001; **50**: 581-585

204 **Marschall HU**, Wagner M, Zollner G, Fickert P, Diczfalusy U, Gumhold J, Silbert D, Fuchsbichler A, Benthin L, Grundström R, Gustafsson U, Sahlin S, Einarsson C, Trauner M. Complementary stimulation of hepatobiliary transport and detoxification systems by rifampicin and ursodeoxycholic acid in humans. *Gastroenterology* 2005; **129**: 476-485

205 **Bachs L**, Parés A, Elena M, Piera C, Rodés J. Comparison of rifampicin with phenobarbitone for treatment of pruritus in biliary cirrhosis. *Lancet* 1989; **1**: 574-576

206 **Hoensch HP**, Balzer K, Dylewizc P, Kirch W, Goebell H, Ohnhaus EE. Effect of rifampicin treatment on hepatic drug metabolism and serum bile acids in patients with primary biliary cirrhosis. *Eur J Clin Pharmacol* 1985; **28**: 475-477

207 **Boelsterli UA**, Rakhit G, Balazs T. Modulation by S-adenosyl-L-methionine of hepatic Na+,K+-ATPase, membrane fluidity, and bile flow in rats with ethinyl estradiol-induced cholestasis. *Hepatology* 1983; **3**: 12-17

208 **Stramentinoli G**, Di Padova C, Gualano M, Rovagnati P, Galli-Kienle M. Ethynylestradiol-induced impairment of bile secretion in the rat: protective effects of S-adenosyl-L-methionine and its implication in estrogen metabolism. *Gastroenterology* 1981; **80**: 154-158

209 **Stramentinoli G**, Gualano M, Di Padova C. Effect of S-adenosyl-L-methionine on ethynylestradiol-induced impairment of bile flow in female rats. *Experientia* 1977; **33**: 1361-1362

210 **Stramentinoli G**, Gualano M, Rovagnati P, Di Padova C. Influence of S-adenosyl-L-methionine on irreversible binding of ethynylestradiol to rat liver microsomes, and its implication in bile secretion. *Biochem Pharmacol* 1979; **28**: 981-984

211 **Frezza M**, Tritapepe R, Pozzato G, Di Padova C. Prevention of S-adenosylmethionine of estrogen-induced hepatobiliary toxicity in susceptible women. *Am J Gastroenterol* 1988; **83**: 1098-1102

212 **Frezza M**, Pozzato G, Chiesa L, Stramentinoli G, di Padova C. Reversal of intrahepatic cholestasis of pregnancy in women after high dose S-adenosyl-L-methionine administration. *Hepatology* 1984; **4**: 274-278

213 **Bonfirraro G**, Chieffi O, Quinti R, Tedesco R, LeGrazie C, Bortolini M. S-adenosyl-L-methionine (SAMe)-induced amelioration of intrahepatic cholestasis of pregnancy. Results of an open study. *Drug Invest* 1990; **2**: 125-128

214 **Frezza M**, Centini G, Cammareri G, Le Grazie C, Di Padova C. S-adenosylmethionine for the treatment of intrahepatic cholestasis of pregnancy. Results of a controlled clinical trial. *Hepatogastroenterology* 1990; **37** Suppl 2: 122-125

215 **Ribalta J**, Reyes H, Gonzalez MC, Iglesias J, Arrese M, Poniachik J, Molina C, Segovia N. S-adenosyl-L-methionine in the treatment of patients with intrahepatic cholestasis of pregnancy: a randomized, double-blind, placebo-controlled study with negative results. *Hepatology* 1991; **13**: 1084-1089

216 **Heikkinen J**, Mäentausta O, Ylöstalo P, Jänne O. Serum bile acid levels in intrahepatic cholestasis of pregnancy during treatment with phenobarbital or cholestyramine. *Eur J Obstet Gynecol Reprod Biol* 1982; **14**: 153-162

217 **Lutz EE**, Margolis AJ. Obstetric hepatosis: treatment with cholestyramine and interim response to steroids. *Obstet Gynecol* 1969; **33**: 64-71

218 **Sadler LC**, Lane M, North R. Severe fetal intracranial haemorrhage during treatment with cholestyramine for intrahepatic cholestasis of pregnancy. *Br J Obstet Gynaecol* 1995; **102**: 169-170

219 **Gylling H**, Riikonen S, Nikkilä K, Savonius H, Miettinen TA. Oral guar gum treatment of intrahepatic cholestasis and pruritus in pregnant women: effects on serum cholestanol and other non-cholesterol sterols. *Eur J Clin Invest* 1998; **28**: 359-363

220 **Riikonen S**, Savonius H, Gylling H, Nikkilä K, Tuomi AM, Miettinen TA. Oral guar gum, a gel-forming dietary fiber relieves pruritus in intrahepatic cholestasis of pregnancy. *Acta Obstet Gynecol Scand* 2000; **79**: 260-264

221 **Kaaja RJ**, Kontula KK, Räihä A, Laatikainen T. Treatment of cholestasis of pregnancy with peroral activated charcoal. A preliminary study. *Scand J Gastroenterol* 1994; **29**: 178-181

222 **Arrese M**, Reyes H. Intrahepatic cholestasis of pregnancy: a past and present riddle. *Ann Hepatol* 2006; **5**: 202-205

223 **Furhoff AK**. Itching in pregnancy. A 15-year follow-up study. *Acta Med Scand* 1974; **196**: 403-410

224 **Ropponen A**, Sund R, Riikonen S, Ylikorkala O, Aittomäki K. Intrahepatic cholestasis of pregnancy as an indicator of liver and biliary diseases: a population-based study. *Hepatology* 2006; **43**: 723-728


**S- Editor** Li LF **L- Editor** Cant MR **E- Editor** Zheng XM

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.15.2067

*World J Gastroenterol* 2009 May 7; 15(17): 2067-2073
***World Journal of Gastroenterology*** ISSN 1007-9327


*EDITORIAL*

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

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Received: February 21, 2009 Revised: March 27, 2009
Accepted: April 3, 2009
Published online: May 7, 2009

## 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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Hoentjen F, van Bodegraven AA. Safety of anti-tumor necrosis factor therapy in inflammatory bowel disease. *World J Gastroenterol* 2009; 15(17): 2067-2073 Available from: URL: http://www.wjgnet.com/1007-9327/15/2067.asp DOI: http://dx.doi.org/10.3748/wjg.15.2067

## 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.

## REFERENCES


1 **Hoentjen F**, Dieleman LA. Pathophysiology of inflammatory bowel diseases. In: Gibson GR, Roberfroid MB, eds. Handbook of prebiotics. Boca Raton: CRC Press, 2008: 341-374

2 **Hommes DW**, Oldenburg B, van Bodegraven AA, van Hogezand RA, de Jong DJ, Romberg-Camps MJ, van der Woude J, Dijkstra G. Guidelines for treatment with infliximab for Crohn's disease. *Neth J Med* 2006; **64**: 219-229

3 **Rutgeerts P**, Van Assche G, Vermeire S. Review article: Infliximab therapy for inflammatory bowel disease--seven years on. *Aliment Pharmacol Ther* 2006; **23**: 451-463

4 **Colombel JF**, Loftus EV Jr, Tremaine WJ, Egan LJ, Harmsen WS, Schleck CD, Zinsmeister AR, Sandborn WJ. The safety profile of infliximab in patients with Crohn's disease: the Mayo clinic experience in 500 patients. *Gastroenterology* 2004; **126**: 19-31

5 **Colombel JF**, Sandborn WJ, Rutgeerts P, Enns R, Hanauer SB, Panaccione R, Schreiber S, Byczkowski D, Li J, Kent JD, Pollack PF. Adalimumab for maintenance of clinical response and remission in patients with Crohn's disease: the CHARM trial. *Gastroenterology* 2007; **132**: 52-65

6 **Schreiber S**, Khaliq-Kareemi M, Lawrance IC, Thomsen OØ, Hanauer SB, McColm J, Bloomfield R, Sandborn WJ. Maintenance therapy with certolizumab pegol for Crohn's disease. *N Engl J Med* 2007; **357**: 239-250

7 **Gómez-Reino JJ**, Carmona L, Angel Descalzo M. Risk of tuberculosis in patients treated with tumor necrosis factor antagonists due to incomplete prevention of reactivation of latent infection. *Arthritis Rheum* 2007; **57**: 756-761

8 **Carmona L**, Gómez-Reino JJ, Rodríguez-Valverde V, Montero D, Pascual-Gómez E, Mola EM, Carreño L, Figueroa M. Effectiveness of recommendations to prevent reactivation of latent tuberculosis infection in patients treated with tumor necrosis factor antagonists. *Arthritis Rheum* 2005; **52**: 1766-1772

9 **Schnitzler F**, Fidder H, Ferrante M, Noman M, Arijs I, Van Assche G, Hoffman I, Van Steen K, Vermeire S, Rutgeerts P. Long-term outcome of treatment with infliximab in 614 patients with Crohn's disease: results from a single-centre cohort. *Gut* 2009; **58**: 492-500

10 **Raval A**, Akhavan-Toyserkani G, Brinker A, Avigan M. Brief communication: characteristics of spontaneous cases of tuberculosis associated with infliximab. *Ann Intern Med* 2007; **147**: 699-702

11 **Dinser R**, Fousse M, Sester U, Albrecht K, Singh M, Köhler H, Müller-Ladner U, Sester M. Evaluation of latent tuberculosis infection in patients with inflammatory arthropathies before treatment with TNF-alpha blocking drugs using a novel flow-cytometric interferon-gamma release assay. *Rheumatology* (Oxford) 2008; **47**: 212-218

12 **Tsiodras S**, Samonis G, Boumpas DT, Kontoyiannis DP. Fungal infections complicating tumor necrosis factor alpha blockade therapy. *Mayo Clin Proc* 2008; **83**: 181-194

13 **Sandborn WJ**, Loftus EV. Balancing the risks and benefits of infliximab in the treatment of inflammatory bowel disease. *Gut* 2004; **53**: 780-782

14 **Toruner M**, Loftus EV Jr, Harmsen WS, Zinsmeister AR, Orenstein R, Sandborn WJ, Colombel JF, Egan LJ. Risk factors for opportunistic infections in patients with inflammatory bowel disease. *Gastroenterology* 2008; **134**: 929-936

15 **Lichtenstein GR**, Feagan BG, Cohen RD, Salzberg BA, Diamond RH, Chen DM, Pritchard ML, Sandborn WJ. Serious infections and mortality in association with therapies for Crohn's disease: TREAT registry. *Clin Gastroenterol Hepatol* 2006; **4**: 621-630

16 **Baert F**, Noman M, Vermeire S, Van Assche G, D' Haens G, Carbonez A, Rutgeerts P. Influence of immunogenicity on the long-term efficacy of infliximab in Crohn's disease. *N Engl J Med* 2003; **348**: 601-608

17 **Van Assche G**, Magdelaine-Beuzelin C, D'Haens G, Baert F, Noman M, Vermeire S, Ternant D, Watier H, Paintaud G, Rutgeerts P. Withdrawal of immunosuppression in Crohn's disease treated with scheduled infliximab maintenance: a randomized trial. *Gastroenterology* 2008; **134**: 1861-1868

18 **Feagan BG**, Panaccione R, Enns RA, Bernstein CN, Ponich TP, Bourdages R, MacIntosh DG, Dallaire C, Cohen A, Fedorak R, Pare P, Bitton A, Saibil F, Anderson F, Donner A, Wong CJ, Zou GY, Vandervoort M, Hopkins M, Greenberg GR. A Randomized Trial of Methotrexate in Combination With Infliximab for the Treatment of Crohn's Disease. *Gastroenterology* 2008; **135**: 294-295

19 **Sandborn W**, Rugeerts P, Reinisch W, Kornbluth A, Lichtiger S, D'Haens G, van der Woude C, Daimond R, Broussard D, Colombel J . SONIC: A randomized, double-blind, controlled trial comparing infliximab and infliximab plus azathioprine to azathioprine in patients with Crohn's disease naive to immunomodulators and biologic therapy. *Am J Gastroenterol* 2008; **103**: S436

20 **Maini RN**, Breedveld FC, Kalden JR, Smolen JS, Davis D, Macfarlane JD, Antoni C, Leeb B, Elliott MJ, Woody JN, Schaible TF, Feldmann M. Therapeutic efficacy of multiple intravenous infusions of anti-tumor necrosis factor alpha monoclonal antibody combined with low-dose weekly methotrexate in rheumatoid arthritis. *Arthritis Rheum* 1998; **41**: 1552-1563

21 **Hanauer SB**, Wagner CL, Bala M, Mayer L, Travers S, Diamond RH, Olson A, Bao W, Rutgeerts P. Incidence and importance of antibody responses to infliximab after maintenance or episodic treatment in Crohn's disease. *Clin Gastroenterol Hepatol* 2004; **2**: 542-553

22 **Sandborn WJ**, Hanauer SB, Rutgeerts P, Fedorak RN, Lukas M, MacIntosh DG, Panaccione R, Wolf D, Kent JD, Bittle B, Li J, Pollack PF. Adalimumab for maintenance treatment of Crohn's disease: results of the CLASSIC II trial. *Gut* 2007; **56**: 1232-1239

23 **Aarden L**, Ruuls SR, Wolbink G. Immunogenicity of anti-tumor necrosis factor antibodies-toward improved methods of anti-antibody measurement. *Curr Opin Immunol* 2008; **20**: 431-435

24 **Bartelds GM**, Wijbrandts CA, Nurmohamed MT, Stapel S, Lems WF, Aarden L, Dijkmans BA, Tak PP, Wolbink GJ. Clinical response to adalimumab: relationship to anti-adalimumab antibodies and serum adalimumab concentrations in rheumatoid arthritis. *Ann Rheum Dis* 2007; **66**: 921-926

25 **West RL**, Zelinkova Z, Wolbink GJ, Kuipers EJ, Stokkers PC, van der Woude CJ. Immunogenicity negatively influences the outcome of adalimumab treatment in Crohn's disease. *Aliment Pharmacol Ther* 2008; **28**: 1122-1126

26 **Cheifetz A**, Smedley M, Martin S, Reiter M, Leone G, Mayer L, Plevy S. The incidence and management of infusion reactions to infliximab: a large center experience. *Am J Gastroenterol* 2003; **98**: 1315-1324

27 **Mayer L**, Young Y. Infusion reactions and their management. *Gastroenterol Clin North Am* 2006; **35**: 857-866
28 **Sandborn WJ**, Feagan BG, Stoinov S, Honiball PJ, Rutgeerts P, Mason D, Bloomfield R, Schreiber S. Certolizumab pegol for the treatment of Crohn's disease. *N Engl J Med* 2007; **357**: 228-238
29 **Ellerin T**, Rubin RH, Weinblatt ME. Infections and anti-tumor necrosis factor alpha therapy. *Arthritis Rheum* 2003; **48**: 3013-3022
30 **Vermeire S**, Noman M, Van Assche G, Baert F, Van Steen K, Esters N, Joossens S, Bossuyt X, Rutgeerts P. Autoimmunity associated with anti-tumor necrosis factor alpha treatment in Crohn's disease: a prospective cohort study. *Gastroenterology* 2003; **125**: 32-39
31 **Benucci M**, Saviola G, Baiardi P, Cammelli E, Manfredi M. Anti-nucleosome antibodies as prediction factor of development of autoantibodies during therapy with three different TNFalpha blocking agents in rheumatoid arthritis. *Clin Rheumatol* 2008; **27**: 91-95
32 **Caramaschi P**, Biasi D, Colombatti M, Pieropan S, Martinelli N, Carletto A, Volpe A, Pacor LM, Bambara LM. Anti-TNFalpha therapy in rheumatoid arthritis and autoimmunity. *Rheumatol Int* 2006; **26**: 209-214
33 **Askling J**, Brandt L, Lapidus A, Karlén P, Björkholm M, Löfberg R, Ekbom A. Risk of haematopoietic cancer in patients with inflammatory bowel disease. *Gut* 2005; **54**: 617-622
34 **Askling J**, Fored CM, Baecklund E, Brandt L, Backlin C, Ekbom A, Sundström C, Bertilsson L, Cöster L, Geborek P, Jacobsson LT, Lindblad S, Lysholm J, Rantapää-Dahlqvist S, Saxne T, Klareskog L, Feltelius N. Haematopoietic malignancies in rheumatoid arthritis: lymphoma risk and characteristics after exposure to tumour necrosis factor antagonists. *Ann Rheum Dis* 2005; **64**: 1414-1420
35 **Bongartz T**, Sutton AJ, Sweeting MJ, Buchan I, Matteson EL, Montori V. Anti-TNF antibody therapy in rheumatoid arthritis and the risk of serious infections and malignancies: systematic review and meta-analysis of rare harmful effects in randomized controlled trials. *JAMA* 2006; **295**: 2275-2285
36 **Lewis JD**, Bilker WB, Brensinger C, Deren JJ, Vaughn DJ, Strom BL. Inflammatory bowel disease is not associated with an increased risk of lymphoma. *Gastroenterology* 2001; **121**: 1080-1087
37 **Jones JL**, Loftus EV Jr. Lymphoma risk in inflammatory bowel disease: is it the disease or its treatment? *Inflamm Bowel Dis* 2007; **13**: 1299-1307
38 **Dayharsh GA**, Loftus EV Jr, Sandborn WJ, Tremaine WJ, Zinsmeister AR, Witzig TE, Macon WR, Burgart LJ. Epstein-Barr virus-positive lymphoma in patients with inflammatory bowel disease treated with azathioprine or 6-mercaptopurine. *Gastroenterology* 2002; **122**: 72-77
39 **Shale M**, Kanfer E, Panaccione R, Ghosh S. Hepatosplenic T cell lymphoma in inflammatory bowel disease. *Gut* 2008; **57**: 1639-1641
40 **Vasiliauskas EA**, Church JA, Silverman N, Barry M, Targan SR, Dubinsky MC. Case report: evidence for transplacental transfer of maternally administered infliximab to the newborn. *Clin Gastroenterol Hepatol* 2006; **4**: 1255-1258
41 **Stengel JZ**, Arnold HL. Is infliximab safe to use while breastfeeding? *World J Gastroenterol* 2008; **14**: 3085-3087
42 **Katz JA**, Antoni C, Keenan GF, Smith DE, Jacobs SJ, Lichtenstein GR. Outcome of pregnancy in women receiving infliximab for the treatment of Crohn's disease and rheumatoid arthritis. *Am J Gastroenterol* 2004; **99**: 2385-2392
43 **Mahadevan U**, Kane S, Sandborn WJ, Cohen RD, Hanson K, Terdiman JP, Binion DG. Intentional infliximab use during pregnancy for induction or maintenance of remission in Crohn's disease. *Aliment Pharmacol Ther* 2005; **21**: 733-738
44 **Mahadevan U**, Terdiman JP, Church J, Vasiliauskas E, Gitis A, Dubinsky MC. Infliximab levels in infants born to women with inflammatory bowel disease. *Gastroenterology* 2007; **132** Suppl 2: A144
45 **Coburn LA**, Wise PE, Schwartz DA. The successful use of adalimumab to treat active Crohn's disease of an ileoanal pouch during pregnancy. *Dig Dis Sci* 2006; **51**: 2045-2047
46 **Mishkin DS**, Van Deinse W, Becker JM, Farraye FA. Successful use of adalimumab (Humira) for Crohn's disease in pregnancy. *Inflamm Bowel Dis* 2006; **12**: 827-828
47 **Johnson D**, Lyons JK, Chambers C. Pregnancy outcomes in women exposed to adalimumab. 73rd annual meeting of the american college of gastroenterology. 2008: 958
48 **Mohan N**, Edwards ET, Cupps TR, Oliverio PJ, Sandberg G, Crayton H, Richert JR, Siegel JN. Demyelination occurring during anti-tumor necrosis factor alpha therapy for inflammatory arthritides. *Arthritis Rheum* 2001; **44**: 2862-2869
49 **Shin IS**, Baer AN, Kwon HJ, Papadopoulos EJ, Siegel JN. Guillain-Barré and Miller Fisher syndromes occurring with tumor necrosis factor alpha antagonist therapy. *Arthritis Rheum* 2006; **54**: 1429-1434
50 **Simsek I**, Erdem H, Pay S, Sobaci G, Dinc A. Optic neuritis occurring with anti-tumour necrosis factor alpha therapy. *Ann Rheum Dis* 2007; **66**: 1255-1258
51 **Menghini VV**, Arora AS. Infliximab-associated reversible cholestatic liver disease. *Mayo Clin Proc* 2001; **76**: 84-86
52 **Moum B**, Konopski Z, Tufteland KF, Jahnsen J. Occurrence of hepatoxicicty and elevated liver enzymes in a Crohn's disease patient treated with infliximab. *Inflamm Bowel Dis* 2007; **13**: 1584-1586
53 **Tobon GJ**, Cañas C, Jaller JJ, Restrepo JC, Anaya JM. Serious liver disease induced by infliximab. *Clin Rheumatol* 2007; **26**: 578-581
54 **Centocor, Inc**. Remicade (infliximab) for IV injection. Full Prescribing Information. IN08520. Malvern, PA: Centocor. Revised December 2008. Accessed April 12, 2009. Available from: URL: http://www.remicade.com/remicade/assets/HCP_PPI.pdf
55 **Millonig G**, Kern M, Ludwiczek O, Nachbaur K, Vogel W. Subfulminant hepatitis B after infliximab in Crohn's disease: need for HBV-screening? *World J Gastroenterol* 2006; **12**: 974-976
56 **Esteve M**, Saro C, González-Huix F, Suarez F, Forné M, Viver JM. Chronic hepatitis B reactivation following infliximab therapy in Crohn's disease patients: need for primary prophylaxis. *Gut* 2004; **53**: 1363-1365
57 **Cepeda EJ**, Williams FM, Ishimori ML, Weisman MH, Reveille JD. The use of anti-tumour necrosis factor therapy in HIV-positive individuals with rheumatic disease. *Ann Rheum Dis* 2008; **67**: 710-712
58 **Fidder H**, Schnitzler F, Ferrante M, Noman M, Katsanos K, Segaert S, Henckaerts L, Van Assche G, Vermeire S, Rutgeerts P. Long-term safety of infliximab for the treatment of inflammatory bowel disease: a single-centre cohort study. *Gut* 2009; **58**: 501-508
59 **Kwon HJ**, Coté TR, Cuffe MS, Kramer JM, Braun MM. Case reports of heart failure after therapy with a tumor necrosis factor antagonist. *Ann Intern Med* 2003; **138**: 807-811


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

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.15.2074

*World J Gastroenterol* 2009 May 7; 15(17): 2074-2077
***World Journal of Gastroenterology*** ISSN 1007-9327


*TOPIC HIGHLIGHT*

**Giada Pietrosi, MD,** *Series Editor*

# Clinical applications of hepatocyte transplantation

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Received: October 24, 2008 Revised: March 14, 2009
Accepted: March 21, 2009
Published online: May 7, 2009

## 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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Pietrosi G, Vizzini GB, Gruttadauria S, Gridelli B. Clinical applications of hepatocyte transplantation. *World J Gastroenterol* 2009; 15(17): 2074-2077 Available from: URL: http://www.wjgnet.com/1007-9327/15/2074.asp DOI: http://dx.doi.org/10.3748/wjg.15.2074

## 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 $2.8 \times 10^7$ 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 $4.7 \times 10^9$ 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 | $1.7 \times 10^7$-$6.0 \times 10^8$ | / | [19] |
| Allotransplant | 10 | | 4/6/3 | |
| Alcohol | 5 | / | 2/3/- | [20] |
| α-1-antitrypsine deficiency | 1 | $2.2 \times 10^7$ | -/1/1 | [4] |
| HCV related | 1 | $7.5 \times 10^6$ | 1/-/- | [4] |
| Other | 3[1] | $5 \times 10^8$-$2 \times 10^9$ | 1/2/2 | [21,22] |
| FHF | 22 | | 13/9/7 | |
| Viral (HSV, HBV) | 6 | $1.2 \times 10^8$-$3 \times 10^{10}$ | 3/3/2 | [4,20,23] |
| Drug-related | 10 | $2.8 \times 10^7$-$3.9 \times 10^{10}$ | 8/2/2 | [4,13,20,22,23] |
| Idiopathic | 4 | $1.8 \times 10^8$-$4 \times 10^9$ | 1/3/3 | [20,22] |
| Other | 2[2] | $1.7 \times 10^8$-$4.9 \times 10^8$ | 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).

($60 \times 10^6$/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.

## ACKNOWLEDGMENTS

The authors would like to thank Warren Blumberg for his help in editing this paper.

## REFERENCES

1 **Fisher RA**, Strom SC. Human hepatocyte transplantation: worldwide results. *Transplantation* 2006; **82**: 441-449

2 **Mitry RR**, Dhawan A, Hughes RD, Bansal S, Lehec S, Terry C, Heaton ND, Karani JB, Mieli-Vergani G, Rela M. One liver,

three recipients: segment IV from split-liver procedures as a source of hepatocytes for cell transplantation. *Transplantation* 2004; **77**: 1614-1616
3 **Habibullah CM**, Syed IH, Qamar A, Taher-Uz Z. Human fetal hepatocyte transplantation in patients with fulminant hepatic failure. *Transplantation* 1994; **58**: 951-952
4 **Strom SC**, Fisher RA, Thompson MT, Sanyal AJ, Cole PE, Ham JM, Posner MP. Hepatocyte transplantation as a bridge to orthotopic liver transplantation in terminal liver failure. *Transplantation* 1997; **63**: 559-569
5 **Sokal EM**. Liver transplantation for inborn errors of liver metabolism. *J Inherit Metab Dis* 2006; **29**: 426-430
6 **Pereira SP**, McCarthy M, Ellis AJ, Wendon J, Portmann B, Rela M, Heaton N, Williams R. Auxiliary partial orthotopic liver transplantation for acute liver failure. *J Hepatol* 1997; **26**: 1010-1017
7 **Allen KJ**, Soriano HE. Liver cell transplantation: the road to clinical application. *J Lab Clin Med* 2001; **138**: 298-312
8 **Horslen SP**, McCowan TC, Goertzen TC, Warkentin PI, Cai HB, Strom SC, Fox IJ. Isolated hepatocyte transplantation in an infant with a severe urea cycle disorder. *Pediatrics* 2003; **111**: 1262-1267
9 **Stephenne X**, Najimi M, Smets F, Reding R, de Ville de Goyet J, Sokal EM. Cryopreserved liver cell transplantation controls ornithine transcarbamylase deficient patient while awaiting liver transplantation. *Am J Transplant* 2005; **5**: 2058-2061
10 **Stephenne X**, Najimi M, Sibille C, Nassogne MC, Smets F, Sokal EM. Sustained engraftment and tissue enzyme activity after liver cell transplantation for argininosuccinate lyase deficiency. *Gastroenterology* 2006; **130**: 1317-1323
11 **Dhawan A**, Mitry RR, Hughes RD. Hepatocyte transplantation for liver-based metabolic disorders. *J Inherit Metab Dis* 2006; **29**: 431-435
12 **Fox IJ**, Chowdhury JR, Kaufman SS, Goertzen TC, Chowdhury NR, Warkentin PI, Dorko K, Sauter BV, Strom SC. Treatment of the Crigler-Najjar syndrome type I with hepatocyte transplantation. *N Engl J Med* 1998; **338**: 1422-1426
13 **Darwish AA**, Sokal E, Stephenne X, Najimi M, de Goyet Jde V, Reding R. Permanent access to the portal system for cellular transplantation using an implantable port device. *Liver Transpl* 2004; **10**: 1213-1215
14 **Ambrosino G**, Varotto S, Strom SC, Guariso G, Franchin E, Miotto D, Caenazzo L, Basso S, Carraro P, Valente ML, D'Amico D, Zancan L, D'Antiga L. Isolated hepatocyte transplantation for Crigler-Najjar syndrome type 1. *Cell Transplant* 2005; **14**: 151-157
15 **Grossman M**, Rader DJ, Muller DW, Kolansky DM, Kozarsky K, Clark BJ 3rd, Stein EA, Lupien PJ, Brewer HB Jr, Raper SE. A pilot study of ex vivo gene therapy for homozygous familial hypercholesterolaemia. *Nat Med* 1995; **1**: 1148-1154
16 **Dhawan A**, Mitry RR, Hughes RD, Lehec S, Terry C, Bansal S, Arya R, Wade JJ, Verma A, Heaton ND, Rela M, Mieli-Vergani G. Hepatocyte transplantation for inherited factor VII deficiency. *Transplantation* 2004; **78**: 1812-1814
17 **Muraca M**, Gerunda G, Neri D, Vilei MT, Granato A, Feltracco P, Meroni M, Giron G, Burlina AB. Hepatocyte transplantation as a treatment for glycogen storage disease type 1a. *Lancet* 2002; **359**: 317-318
18 **Sokal EM**, Smets F, Bourgois A, Van Maldergem L, Buts JP, Reding R, Bernard Otte J, Evrard V, Latinne D, Vincent MF, Moser A, Soriano HE. Hepatocyte transplantation in a 4-year-old girl with peroxisomal biogenesis disease: technique, safety, and metabolic follow-up. *Transplantation* 2003; **76**: 735-738
19 **Mito M**, Kusano M, Kawaura Y. Hepatocyte transplantation in man. *Transplant Proc* 1992; **24**: 3052-3053
20 **Strom SC**, Chowdhury JR, Fox IJ. Hepatocyte transplantation for the treatment of human disease. *Semin Liver Dis* 1999; **19**: 39-48
21 **Baccarani U**, Adani GL, Sanna A, Avellini C, Sainz-Barriga M, Lorenzin D, Montanaro D, Gasparini D, Risaliti A, Donini A, Bresadola F. Portal vein thrombosis after intraportal hepatocytes transplantation in a liver transplant recipient. *Transpl Int* 2005; **18**: 750-754
22 **Soriano HE**, Wood RP, Kang DC, Ozaki CF, Finegold MJ, Bischoff FC, Reid BS, Ferry GD. Hepatocellular transplantation (HCT) in children with fulmina nt liver failure (FLF). *Hepatology* 1997; **26**: 239A
23 **Bilir BM**, Guinette D, Karrer F, Kumpe DA, Krysl J, Stephens J, McGavran L, Ostrowska A, Durham J. Hepatocyte transplantation in acute liver failure. *Liver Transpl* 2000; **6**: 32-40
24 **Bachoud-Levi AC**, Remy P, Nguyen JP, Brugieres P, Lefaucheur JP, Bourdet C, Baudic S, Gaura V, Maison P, Haddad B, Boisse MF, Grandmougin T, Jeny R, Bartolomeo P, Dalla Barba G, Degos JD, Lisovoski F, Ergis AM, Pailhous E, Cesaro P, Hantraye P, Peschanski M. Motor and cognitive improvements in patients with Huntington's disease after neural transplantation. *Lancet* 2000; **356**: 1975-1979
25 **Lupp A**, Danz M, Muller D. Evaluation of 2-year-old intrasplenic fetal liver tissue transplants in rats. *Cell Transplant* 2003; **12**: 423-438

**S- Editor** Li LF **L- Editor** Negro F **E- Editor** Ma WH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.15.2078

*World J Gastroenterol* 2009 May 7; 15(17): 2078-2080
***World Journal of Gastroenterology*** ISSN 1007-9327


*OBSERVATION*

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# *Tropheryma whipplei* infection

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Received: March 11, 2009 Revised: April 7, 2009
Accepted: April 14, 2009
Published online: May 7, 2009

## 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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Freeman HJ. *Tropheryma whipplei* infection. *World J Gastroenterol* 2009; 15(17): 2078-2080 Available from: URL: http://www.wjgnet.com/1007-9327/15/2078.asp DOI: http://dx.doi.org/10.3748/wjg.15.2078

## 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].

## REFERENCES

1 **Raoult D**, Birg ML, La Scola B, Fournier PE, Enea M, Lepidi H, Roux V, Piette JC, Vandenesch F, Vital-Durand D, Marrie TJ. Cultivation of the bacillus of Whipple's disease. *N Engl J Med* 2000; **342**: 620-625

2 **Bentley SD**, Maiwald M, Murphy LD, Pallen MJ, Yeats CA,

Dover LG, Norbertczak HT, Besra GS, Quail MA, Harris DE, von Herbay A, Goble A, Rutter S, Squares R, Squares S, Barrell BG, Parkhill J, Relman DA. Sequencing and analysis of the genome of the Whipple's disease bacterium Tropheryma whipplei. *Lancet* 2003; **361**: 637-644

3 **Raoult D**, Ogata H, Audic S, Robert C, Suhre K, Drancourt M, Claverie JM. Tropheryma whipplei Twist: a human pathogenic Actinobacteria with a reduced genome. *Genome Res* 2003; **13**: 1800-1809

4 **Boulos A**, Rolain JM, Raoult D. Antibiotic susceptibility of Tropheryma whipplei in MRC5 cells. *Antimicrob Agents Chemother* 2004; **48**: 747-752

5 **Boulos A**, Rolain JM, Mallet MN, Raoult D. Molecular evaluation of antibiotic susceptibility of Tropheryma whipplei in axenic medium. *J Antimicrob Chemother* 2005; **55**: 178-181

6 **Fenollar F**, Puéchal X, Raoult D. Whipple's disease. *N Engl J Med* 2007; **356**: 55-66

7 **Marth T**, Schneider T. Whipple disease. *Curr Opin Gastroenterol* 2008; **24**: 141-148

8 **Cohen AS**, Schimmel EM, Holt PR, Isselbacher KJ. Ultrastructural abnormalities in Whipple's disease. *Proc Soc Exp Biol Med* 1960; **105**: 411-414

9 **Yardley JH**, Hendrix TR. Combined electron and light microscopy in Whipple's disease. Demonstration of "bacillary bodies" in the intestine. *Bull Johns Hopkins Hosp* 1961; **109**: 80-98

10 **Lepidi H**, Fenollar F, Dumler JS, Gauduchon V, Chalabreysse L, Bammert A, Bonzi MF, Thivolet-Béjui F, Vandenesch F, Raoult D. Cardiac valves in patients with Whipple endocarditis: microbiological, molecular, quantitative histologic, and immunohistochemical studies of 5 patients. *J Infect Dis* 2004; **190**: 935-945

11 **Maiwald M**, Schuhmacher F, Ditton HJ, von Herbay A. Environmental occurrence of the Whipple's disease bacterium (Tropheryma whippelii). *Appl Environ Microbiol* 1998; **64**: 760-762

12 **Schöniger-Hekele M**, Petermann D, Weber B, Müller C. Tropheryma whipplei in the environment: survey of sewage plant influxes and sewage plant workers. *Appl Environ Microbiol* 2007; **73**: 2033-2035

13 **Moos V**, Kunkel D, Marth T, Feurle GE, LaScola B, Ignatius R, Zeitz M, Schneider T. Reduced peripheral and mucosal Tropheryma whipplei-specific Th1 response in patients with Whipple's disease. *J Immunol* 2006; **177**: 2015-2022

14 **Fleming JL**, Wiesner RH, Shorter RG. Whipple's disease: clinical, biochemical, and histopathologic features and assessment of treatment in 29 patients. *Mayo Clin Proc* 1988; **63**: 539-551

15 **Maizel H**, Ruffin JM, Dobbins WO 3rd. Whipple's disease: a review of 19 patients from one hospital and a review of the literature since 1950. 1970. *Medicine* (Baltimore) 1993; **72**: 343-355

16 **Armelao F**, Portolan F, Togni R. Mosaic-patterned and scalloped duodenal mucosa in Whipple's disease. *Clin Gastroenterol Hepatol* 2008; **6**: A32

17 **Lewin KJ**, Riddell RH, Weinstein WM. Gastrointestinal pathology and its clinical implications. New York: Igaku-Shoin, 1992: 779-782

18 **Keinath RD**, Merrell DE, Vlietstra R, Dobbins WO 3rd. Antibiotic treatment and relapse in Whipple's disease. Long-term follow-up of 88 patients. *Gastroenterology* 1985; **88**: 1867-1873

19 **Gerard A**, Sarrot-Reynauld F, Liozon E, Cathebras P, Besson G, Robin C, Vighetto A, Mosnier JF, Durieu I, Vital Durand D, Rousset H. Neurologic presentation of Whipple disease: report of 12 cases and review of the literature. *Medicine* (Baltimore) 2002; **81**: 443-457

20 **Mendel E**, Khoo LT, Go JL, Hinton D, Zee CS, Apuzzo ML. Intracerebral Whipple's disease diagnosed by stereotactic biopsy: a case report and review of the literature. *Neurosurgery* 1999; **44**: 203-209

21 **Panegyres PK**, Edis R, Beaman M, Fallon M. Primary Whipple's disease of the brain: characterization of the clinical syndrome and molecular diagnosis. *QJM* 2006; **99**: 609-623

22 **von Herbay A**, Ditton HJ, Schuhmacher F, Maiwald M. Whipple's disease: staging and monitoring by cytology and polymerase chain reaction analysis of cerebrospinal fluid. *Gastroenterology* 1997; **113**: 434-441

23 **Avila MP**, Jalkh AE, Feldman E, Trempe CL, Schepens CL. Manifestations of Whipple's disease in the posterior segment of the eye. *Arch Ophthalmol* 1984; **102**: 384-390

24 **Houpikian P**, Raoult D. Blood culture-negative endocarditis in a reference center: etiologic diagnosis of 348 cases. *Medicine* (Baltimore) 2005; **84**: 162-173

25 **Fenollar F**, Laouira S, Lepidi H, Rolain JM, Raoult D. Value of Tropheryma whipplei quantitative polymerase chain reaction assay for the diagnosis of Whipple disease: usefulness of saliva and stool specimens for first-line screening. *Clin Infect Dis* 2008; **47**: 659-667

26 **Baisden BL**, Lepidi H, Raoult D, Argani P, Yardley JH, Dumler JS. Diagnosis of Wihipple disease by immunohistochemical analysis: a sensitive and specific method for the detection of Tropheryma whipplei (the Whipple bacillus) in paraffin-embedded tissue. *Am J Clin Pathol* 2002; **118**: 742-748

27 **Kowalczewska M**, Raoult D. Advances in Tropheryma whipplei research: the rush to find biomarkers for Whipple's disease. *Future Microbiol* 2007; **2**: 631-642

28 **Caples SM**, Petrovic LM, Ryu JH. Successful treatment of Whipple disease diagnosed 36 years after symptom onset. *Mayo Clin Proc* 2001; **76**: 1063-1066

29 **Schneider T**, Stallmach A, von Herbay A, Marth T, Strober W, Zeitz M. Treatment of refractory Whipple disease with interferon-gamma. *Ann Intern Med* 1998; **129**: 875-877

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

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.15.2081

*World J Gastroenterol* 2009 May 7; 15(17): 2081-2088
***World Journal of Gastroenterology*** ISSN 1007-9327


*REVIEW*

# Importance of nutrition in inflammatory bowel disease

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Received: December 18, 2008 Revised: March 30, 2009
Accepted: April 6, 2009
Published online: May 7, 2009

## 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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Lucendo AJ, De Rezende LC. Importance of nutrition in inflammatory bowel disease. *World J Gastroenterol* 2009; 15(17): 2081-2088 Available from: URL: http://www.wjgnet.com/1007-9327/15/2081.asp DOI: http://dx.doi.org/10.3748/wjg.15.2081

## 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.

## REFERENCES


1 **Podolsky DK**. Inflammatory bowel disease. *N Engl J Med* 2002; **347**: 417-429
2 **Hugot JP**, Chamaillard M, Zouali H, Lesage S, Cézard JP, Belaiche J, Almer S, Tysk C, O'Morain CA, Gassull M, Binder V, Finkel Y, Cortot A, Modigliani R, Laurent-Puig P, Gower-Rousseau C, Macry J, Colombel JF, Sahbatou M, Thomas G. Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. *Nature* 2001; **411**: 599-603
3 **Ogura Y**, Bonen DK, Inohara N, Nicolae DL, Chen FF, Ramos R, Britton H, Moran T, Karaliuskas R, Duerr RH, Achkar JP, Brant SR, Bayless TM, Kirschner BS, Hanauer SB, Nuñez G, Cho JH. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature* 2001; **411**: 603-606
4 **Bernstein CN**, Shanahan F. Disorders of a modern lifestyle: reconciling the epidemiology of inflammatory bowel diseases. *Gut* 2008; **57**: 1185-1191
5 **Bernstein CN**, Rawsthorne P, Cheang M, Blanchard JF. A population-based case control study of potential risk factors for IBD. *Am J Gastroenterol* 2006; **101**: 993-1002
6 **Cashman KD**, Shanahan F. Is nutrition an aetiological factor for inflammatory bowel disease? *Eur J Gastroenterol Hepatol* 2003; **15**: 607-613
7 **Campos FG**, Waitzberg DL, Teixeira MG, Mucerino DR, Kiss DR, Habr-Gama A. Pharmacological nutrition in inflammatory bowel diseases. *Nutr Hosp* 2003; **18**: 57-64
8 **Shanahan F**. Inflammatory bowel disease: immunodiagnostics, immunotherapeutics, and ecotherapeutics. *Gastroenterology* 2001; **120**: 622-635
9 **Acheson ED**, Truelove SC. Early weaning in the aetiology of ulcerative colitis. A study of feeding in infancy in cases and controls. *Br Med J* 1961; **2**: 929-933
10 **Whorwell PJ**, Holdstock G, Whorwell GM, Wright R. Bottle feeding, early gastroenteritis, and inflammatory bowel disease. *Br Med J* 1979; **1**: 382
11 **Corrao G**, Tragnone A, Caprilli R, Trallori G, Papi C, Andreoli A, Di Paolo M, Riegler G, Rigo GP, Ferraù O, Mansi C, Ingrosso M, Valpiani D. Risk of inflammatory bowel disease attributable to smoking, oral contraception and breastfeeding in Italy: a nationwide case-control study. Cooperative Investigators of the Italian Group for the Study of the Colon and the Rectum (GISC). *Int J Epidemiol* 1998; **27**: 397-404
12 **Bergstrand O**, Hellers G. Breast-feeding during infancy in patients who later develop Crohn's disease. *Scand J Gastroenterol* 1983; **18**: 903-906
13 **Koletzko S**, Sherman P, Corey M, Griffiths A, Smith C. Role of infant feeding practices in development of Crohn's disease in childhood. *BMJ* 1989; **298**: 1617-1618
14 **Binder JH**, Gryboski JD, Thayer WR Jr, Spiro HM. Intolerance to milk in ulcerative colitis. A preliminary report. *Am J Dig Dis* 1966; **11**: 858-864
15 **Lerner A**, Rossi TM, Park B, Albini B, Lebenthal E. Serum antibodies to cow's milk proteins in pediatric inflammatory bowel disease: Crohn's disease vs. ulcerative colitis. *Acta Paediatr Scand* 1989; **78**: 81-86
16 **Knoflach P**, Park BH, Cunningham R, Weiser MM, Albini B. Serum antibodies to cow's milk proteins in ulcerative colitis and Crohn's disease. *Gastroenterology* 1987; **92**: 479-485
17 **Beaudry M**, Dufour R, Marcoux S. Relation between infant feeding and infections during the first six months of life. *J Pediatr* 1995; **126**: 191-197
18 **Duffy LC**, Byers TE, Riepenhoff-Talty M, La Scolea LJ, Zielezny M, Ogra PL. The effects of infant feeding on rotavirus-induced gastroenteritis: a prospective study. *Am J Public Health* 1986; **76**: 259-263
19 **Howie PW**, Forsyth JS, Ogston SA, Clark A, Florey CD. Protective effect of breast feeding against infection. *BMJ* 1990; **300**: 11-16
20 **Pittard WB 3rd**. Breast milk immunology. A frontier in infant nutrition. *Am J Dis Child* 1979; **133**: 83-87
21 **Pittard WB 3rd**, Bill K. Immunoregulation by breast milk cells. *Cell Immunol* 1979; **42**: 437-441
22 **Bernt KM**, Walker WA. Human milk as a carrier of biochemical messages. *Acta Paediatr Suppl* 1999; **88**: 27-41
23 **Carver JD**, Barness LA. Trophic factors for the gastrointestinal tract. *Clin Perinatol* 1996; **23**: 265-285
24 **Hermon-Taylor J**. Protagonist. Mycobacterium avium subspecies paratuberculosis is a cause of Crohn's disease. *Gut* 2001; **49**: 755-756
25 **Feller M**, Huwiler K, Stephan R, Altpeter E, Shang A, Furrer H, Pfyffer GE, Jemmi T, Baumgartner A, Egger M. Mycobacterium avium subspecies paratuberculosis and Crohn's disease: a systematic review and meta-analysis. *Lancet Infect Dis* 2007; **7**: 607-613
26 **Harris JE**, Lammerding AM. Crohn's disease and Mycobacterium avium subsp. paratuberculosis: current issues. *J Food Prot* 2001; **64**: 2103-2110
27 **Sartor RB**. Does Mycobacterium avium subspecies paratuberculosis cause Crohn's disease? *Gut* 2005; **54**: 896-898
28 **Martini GA**, Brandes JW. Increased consumption of refined carbohydrates in patients with Crohn's disease. *Klin Wochenschr* 1976; **54**: 367-371
29 **Miller B**, Fervers F, Rohbeck R, Strohmeyer G. [Sugar consumption in patients with Crohn's disease] *Verh Dtsch Ges Inn Med* 1976; **82** Pt 1: 922-924
30 **Reif S**, Klein I, Lubin F, Farbstein M, Hallak A, Gilat T. Pre-illness dietary factors in inflammatory bowel disease. *Gut* 1997; **40**: 754-760
31 **Mayberry JF**, Rhodes J, Newcombe RG. Increased sugar consumption in Crohn's disease. *Digestion* 1980; **20**: 323-326
32 **Geerling BJ**, Stockbrügger RW, Brummer RJ. Nutrition and inflammatory bowel disease: an update. *Scand J Gastroenterol Suppl* 1999; **230**: 95-105
33 **Thornton JR**, Emmett PM, Heaton KW. Smoking, sugar, and inflammatory bowel disease. *Br Med J* (Clin Res Ed) 1985; **290**: 1786-1787
34 **Husain A**, Korzenik JR. Nutritional issues and therapy in inflammatory bowel disease. *Semin Gastrointest Dis* 1998; **9**: 21-30
35 **Panza E**, Franceschi S, La Vecchia C. Dietary factors in the aetiology of inflammatory bowel disease. *Ital J Gastroenterol* 1987; **19**: 205-209
36 **Russel MG**, Engels LG, Muris JW, Limonard CB, Volovics A, Brummer RJ, Stockbrügger RW. Modern life' in the epidemiology of inflammatory bowel disease: a case-control study with special emphasis on nutritional factors. *Eur J Gastroenterol Hepatol* 1998; **10**: 243-249
37 **Kasper H**, Sommer H. Dietary fiber and nutrient intake in Crohn's disease. *Am J Clin Nutr* 1979; **32**: 1898-1901
38 **Thornton JR**, Emmett PM, Heaton KW. Diet and Crohn's disease: characteristics of the pre-illness diet. *Br Med J* 1979; **2**: 762-764
39 **Bianchi Porro G**, Panza E. Smoking, sugar, and inflammatory bowel disease. *Br Med J* (Clin Res Ed) 1985; **291**: 971-972
40 **Rawcliffe PM**, Truelove SC. Breakfast and Crohn's disease--I. *Br Med J* 1978; **2**: 539-540
41 **Ritchie JK**, Wadsworth J, Lennard-Jones JE, Rogers E. Controlled multicentre therapeutic trial of an unrefined carbohydrate, fibre rich diet in Crohn's disease. *Br Med J* (Clin Res Ed) 1987; **295**: 517-520
42 **Guthy E**. [Crohn's disease and nutritional lipids. Hypothesis on etiology of regional enteritis] *Dtsch Med Wochenschr* 1982; **107**: 71-73
43 Dietary and other risk factors of ulcerative colitis. A case-control study in Japan. Epidemiology Group of the Research Committee of Inflammatory Bowel Disease in Japan. *J Clin Gastroenterol* 1994; **19**: 166-171
44 **Persson PG**, Ahlbom A, Hellers G. Diet and inflammatory

bowel disease: a case-control study. *Epidemiology* 1992; **3**: 47-52
45 **Geerling BJ**, Dagnelie PC, Badart-Smook A, Russel MG, Stockbrügger RW, Brummer RJ. Diet as a risk factor for the development of ulcerative colitis. *Am J Gastroenterol* 2000; **95**: 1008-1013
46 **Shoda R**, Matsueda K, Yamato S, Umeda N. Epidemiologic analysis of Crohn disease in Japan: increased dietary intake of n-6 polyunsaturated fatty acids and animal protein relates to the increased incidence of Crohn disease in Japan. *Am J Clin Nutr* 1996; **63**: 741-745
47 **Kromann N**, Green A. Epidemiological studies in the Upernavik district, Greenland. Incidence of some chronic diseases 1950-1974. *Acta Med Scand* 1980; **208**: 401-406
48 **Bang HO**, Dyerberg J, Sinclair HM. The composition of the Eskimo food in north western Greenland. *Am J Clin Nutr* 1980; **33**: 2657-2661
49 **Grimble RF**, Tappia PS. Modulation of pro-inflammatory cytokine biology by unsaturated fatty acids. *Z Ernahrungswiss* 1998; **37** Suppl 1: 57-65
50 **Jeffery NM**, Newsholme EA, Calder PC. Level of polyunsaturated fatty acids and the n-6 to n-3 polyunsaturated fatty acid ratio in the rat diet alter serum lipid levels and lymphocyte functions. *Prostaglandins Leukot Essent Fatty Acids* 1997; **57**: 149-160
51 **Chapman MA**, Grahn MF, Boyle MA, Hutton M, Rogers J, Williams NS. Butyrate oxidation is impaired in the colonic mucosa of sufferers of quiescent ulcerative colitis. *Gut* 1994; **35**: 73-76
52 **Kim YI**. Short-chain fatty acids in ulcerative colitis. *Nutr Rev* 1998; **56**: 17-24
53 **Simpson EJ**, Chapman MA, Dawson J, Berry D, Macdonald IA, Cole A. In vivo measurement of colonic butyrate metabolism in patients with quiescent ulcerative colitis. *Gut* 2000; **46**: 73-77
54 **Gee MI**, Grace MG, Wensel RH, Sherbaniuk RW, Thomson AB. Nutritional status of gastroenterology outpatients: comparison of inflammatory bowel disease with functional disorders. *J Am Diet Assoc* 1985; **85**: 1591-1599
55 **Goh J**, O'Morain CA. Review article: nutrition and adult inflammatory bowel disease. *Aliment Pharmacol Ther* 2003; **17**: 307-320
56 **Han PD**, Burke A, Baldassano RN, Rombeau JL, Lichtenstein GR. Nutrition and inflammatory bowel disease. *Gastroenterol Clin North Am* 1999; **28**: 423-443, ix
57 **Gassull MA**, Cabré E. Nutrition in inflammatory bowel disease. *Curr Opin Clin Nutr Metab Care* 2001; **4**: 561-569
58 **Driscoll RH Jr**, Rosenberg IH. Total parenteral nutrition in inflammatory bowel disease. *Med Clin North Am* 1978; **62**: 185-201
59 **Al-Jaouni R**, Hébuterne X, Pouget I, Rampal P. Energy metabolism and substrate oxidation in patients with Crohn's disease. *Nutrition* 2000; **16**: 173-178
60 **Mingrone G**, Capristo E, Greco AV, Benedetti G, De Gaetano A, Tataranni PA, Gasbarrini G. Elevated diet-induced thermogenesis and lipid oxidation rate in Crohn disease. *Am J Clin Nutr* 1999; **69**: 325-330
61 **Klein S**, Meyers S, O'Sullivan P, Barton D, Leleiko N, Janowitz HD. The metabolic impact of active ulcerative colitis. Energy expenditure and nitrogen balance. *J Clin Gastroenterol* 1988; **10**: 34-40
62 **Cabré E**, Gassull MA. Nutrition in inflammatory bowel disease: impact on disease and therapy. *Curr Opin Gastroenterol* 2001; **17**: 342-349
63 **García-Manzanares Vázquez de Agredos A**, Álvarez Hernandez J, Maqueda Villaizan E. Soporte nutricional en la enfermedad inflamatoria intestinal. In: Bellido D, De Luis D, editors. Manual de Nutrición y Metabolismo. Madrid: Díaz de Santos, 2006: 333-348
64 **Gassull MA**. Review article: the role of nutrition in the treatment of inflammatory bowel disease. *Aliment Pharmacol Ther* 2004; **20** Suppl 4: 79-83
65 **Bjarnason I**, Macpherson A, Mackintosh C, Buxton-Thomas M, Forgacs I, Moniz C. Reduced bone density in patients with inflammatory bowel disease. *Gut* 1997; **40**: 228-233
66 **Jahnsen J**, Falch JA, Mowinckel P, Aadland E. Bone mineral density in patients with inflammatory bowel disease: a population-based prospective two-year follow-up study. *Scand J Gastroenterol* 2004; **39**: 145-153
67 **Tilg H**, Moschen AR, Kaser A, Pines A, Dotan I. Gut, inflammation and osteoporosis: basic and clinical concepts. *Gut* 2008; **57**: 684-694
68 **Pollak RD**, Karmeli F, Eliakim R, Ackerman Z, Tabb K, Rachmilewitz D. Femoral neck osteopenia in patients with inflammatory bowel disease. *Am J Gastroenterol* 1998; **93**: 1483-1490
69 **Compston JE**. Review article: osteoporosis, corticosteroids and inflammatory bowel disease. *Aliment Pharmacol Ther* 1995; **9**: 237-250
70 **Lashner BA**. Red blood cell folate is associated with the development of dysplasia and cancer in ulcerative colitis. *J Cancer Res Clin Oncol* 1993; **119**: 549-554
71 **Lashner BA**, Heidenreich PA, Su GL, Kane SV, Hanauer SB. Effect of folate supplementation on the incidence of dysplasia and cancer in chronic ulcerative colitis. A case-control study. *Gastroenterology* 1989; **97**: 255-259
72 **Lashner BA**, Provencher KS, Seidner DL, Knesebeck A, Brzezinski A. The effect of folic acid supplementation on the risk for cancer or dysplasia in ulcerative colitis. *Gastroenterology* 1997; **112**: 29-32
73 **Talbot RW**, Heppell J, Dozois RR, Beart RW Jr. Vascular complications of inflammatory bowel disease. *Mayo Clin Proc* 1986; **61**: 140-145
74 **Mahmud N**, Molloy A, McPartlin J, Corbally R, Whitehead AS, Scott JM, Weir DG. Increased prevalence of methylene tetrahydrofolate reductase C677T variant in patients with inflammatory bowel disease, and its clinical implications. *Gut* 1999; **45**: 389-394
75 **McClain C**, Soutor C, Zieve L. Zinc deficiency: a complication of Crohn's disease. *Gastroenterology* 1980; **78**: 272-279
76 **Kruis W**, Rindfleisch GE, Weinzierl M. Zinc deficiency as a problem in patients with Crohn's disease and fistula formation. *Hepatogastroenterology* 1985; **32**: 133-134
77 **Reimund JM**, Arondel Y, Escalin G, Finck G, Baumann R, Duclos B. Immune activation and nutritional status in adult Crohn's disease patients. *Dig Liver Dis* 2005; **37**: 424-431
78 **Reimund JM**, Hirth C, Koehl C, Baumann R, Duclos B. Antioxidant and immune status in active Crohn's disease. A possible relationship. *Clin Nutr* 2000; **19**: 43-48
79 **Motil KJ**, Grand RJ, Davis-Kraft L, Ferlic LL, Smith EO. Growth failure in children with inflammatory bowel disease: a prospective study. *Gastroenterology* 1993; **105**: 681-691
80 **Wong SC**, Macrae VE, McGrogan P, Ahmed SF. The role of pro-inflammatory cytokines in inflammatory bowel disease growth retardation. *J Pediatr Gastroenterol Nutr* 2006; **43**: 144-155
81 **Ahmed SF**, Wong JS, McGrogan P. Improving growth in children with inflammatory bowel disease. *Horm Res* 2007; **68** Suppl 5: 117-121
82 **Menchén L**, Ripoll C, Bretón I, Moreno C, de la Cuerda C, Camblor M, García-Peris P, González-Lara V, Cos E. [Osteoporosis and inflammatory bowel disease] *Nutr Hosp* 2005; **20**: 26-37
83 **Lochs H**, Steinhardt HJ, Klaus-Wentz B, Zeitz M, Vogelsang H, Sommer H, Fleig WE, Bauer P, Schirrmeister J, Malchow H. Comparison of enteral nutrition and drug treatment in active Crohn's disease. Results of the European Cooperative Crohn's Disease Study. IV. *Gastroenterology* 1991; **101**: 881-888
84 **Heuschkel R**. Enteral nutrition in crohn disease: more than just calories. *J Pediatr Gastroenterol Nutr* 2004; **38**: 239-241
85 **Voitk AJ**, Echave V, Feller JH, Brown RA, Gurd FN. Experience with elemental diet in the treatment of

inflammatory bowel disease. Is this primary therapy? *Arch Surg* 1973; **107**: 329-333
86 **O'Moráin C**, Segal AW, Levi AJ. Elemental diet as primary treatment of acute Crohn's disease: a controlled trial. *Br Med J* (Clin Res Ed) 1984; **288**: 1859-1862
87 **Fernández-Banares F**, Cabré E, Esteve-Comas M, Gassull MA. How effective is enteral nutrition in inducing clinical remission in active Crohn's disease? A meta-analysis of the randomized clinical trials. *JPEN J Parenter Enteral Nutr* 1995; **19**: 356-364
88 **Heuschkel RB**, Menache CC, Megerian JT, Baird AE. Enteral nutrition and corticosteroids in the treatment of acute Crohn's disease in children. *J Pediatr Gastroenterol Nutr* 2000; **31**: 8-15
89 **Wilschanski M**, Sherman P, Pencharz P, Davis L, Corey M, Griffiths A. Supplementary enteral nutrition maintains remission in paediatric Crohn's disease. *Gut* 1996; **38**: 543-548
90 **Hiwatashi N**. Enteral nutrition for Crohn's disease in Japan. *Dis Colon Rectum* 1997; **40**: S48-S53
91 **Akobeng AK**, Thomas AG. Enteral nutrition for maintenance of remission in Crohn's disease. *Cochrane Database Syst Rev* 2007; CD005984
92 **Ikeuchi H**, Yamamura T, Nakano H, Kosaka T, Shimoyama T, Fukuda Y. Efficacy of nutritional therapy for perforating and non-perforating Crohn's disease. *Hepatogastroenterology* 2004; **51**: 1050-1052
93 **Zachos M**, Tondeur M, Griffiths AM. Enteral nutritional therapy for inducing remission of Crohn's disease. *Cochrane Database Syst Rev* 2001; CD000542
94 **González-Huix F**, de León R, Fernández-Bañares F, Esteve M, Cabré E, Acero D, Abad-Lacruz A, Figa M, Guilera M, Planas R. Polymeric enteral diets as primary treatment of active Crohn's disease: a prospective steroid controlled trial. *Gut* 1993; **34**: 778-782
95 **Gassull MA**, Fernández-Bañares F, Cabré E, Papo M, Giaffer MH, Sánchez-Lombraña JL, Richart C, Malchow H, González-Huix F, Esteve M. Fat composition may be a clue to explain the primary therapeutic effect of enteral nutrition in Crohn's disease: results of a double blind randomised multicentre European trial. *Gut* 2002; **51**: 164-168
96 **Belluzzi A**, Brignola C, Campieri M, Pera A, Boschi S, Miglioli M. Effect of an enteric-coated fish-oil preparation on relapses in Crohn's disease. *N Engl J Med* 1996; **334**: 1557-1560
97 **Turner D**, Zlotkin SH, Shah PS, Griffiths AM. Omega 3 fatty acids (fish oil) for maintenance of remission in Crohn's disease. *Cochrane Database Syst Rev* 2007; CD006320
98 **Turner D**, Steinhart AH, Griffiths AM. Omega 3 fatty acids (fish oil) for maintenance of remission in ulcerative colitis. *Cochrane Database Syst Rev* 2007; CD006443
99 **Gupta P**, Andrew H, Kirschner BS, Guandalini S. Is lactobacillus GG helpful in children with Crohn's disease? Results of a preliminary, open-label study. *J Pediatr Gastroenterol Nutr* 2000; **31**: 453-457
100 **Gionchetti P**, Lammers KM, Rizzello F, Campieri M. Probiotics and barrier function in colitis. *Gut* 2005; **54**: 898-900
101 **Packey CD**, Sartor RB. Interplay of commensal and pathogenic bacteria, genetic mutations, and immunoregulatory defects in the pathogenesis of inflammatory bowel diseases. *J Intern Med* 2008; **263**: 597-606
102 **Sartor RB**. Microbial influences in inflammatory bowel diseases. *Gastroenterology* 2008; **134**: 577-594
103 **Bibiloni R**, Fedorak RN, Tannock GW, Madsen KL, Gionchetti P, Campieri M, De Simone C, Sartor RB. VSL#3 probiotic-mixture induces remission in patients with active ulcerative colitis. *Am J Gastroenterol* 2005; **100**: 1539-1546
104 **Chapman TM**, Plosker GL, Figgitt DP. VSL#3 probiotic mixture: a review of its use in chronic inflammatory bowel diseases. *Drugs* 2006; **66**: 1371-1387
105 **Chapman MA**. The role of the colonic flora in maintaining a healthy large bowel mucosa. *Ann R Coll Surg Engl* 2001; **83**: 75-80
106 **Segain JP**, Raingeard de la Blétière D, Bourreille A, Leray V, Gervois N, Rosales C, Ferrier L, Bonnet C, Blottière HM, Galmiche JP. Butyrate inhibits inflammatory responses through NFkappaB inhibition: implications for Crohn's disease. *Gut* 2000; **47**: 397-403
107 **Steinhart AH**, Hiruki T, Brzezinski A, Baker JP. Treatment of left-sided ulcerative colitis with butyrate enemas: a controlled trial. *Aliment Pharmacol Ther* 1996; **10**: 729-736
108 **Assumpção IR**, Rodrigues M, Barbieri D. [Treatment of unspecific ulcerative rectocolitis in a child with enemas containing butyrate. Case report] *Arq Gastroenterol* 1999; **36**: 238-243
109 **Greenberg GR**, Fleming CR, Jeejeebhoy KN, Rosenberg IH, Sales D, Tremaine WJ. Controlled trial of bowel rest and nutritional support in the management of Crohn's disease. *Gut* 1988; **29**: 1309-1315
110 **Lochs H**, Meryn S, Marosi L, Ferenci P, Hörtnagl H. Has total bowel rest a beneficial effect in the treatment of Crohn's disease? *Clin Nutr* 1983; **2**: 61-64
111 **Cabré Gelada E**, Gassull Duró MA. [Parenteral nutrition versus enteral nutrition. When and why?] *Med Clin* (Barc) 1991; **96**: 692-693
112 **Duerksen DR**, Nehra V, Bistrian BR, Blackburn GL. Appropriate nutritional support in acute and complicated Crohn's disease. *Nutrition* 1998; **14**: 462-465
113 **Khanna MP**, Gordon PH. Gastrocolic fistulization in Crohn's disease: a case report and a review of the literature. *Can J Surg* 2000; **43**: 53-56

**S- Editor** Li LF **L- Editor** Logan S **E- Editor** Zheng XM

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.15.2089

*World J Gastroenterol* 2009 May 7; 15(17): 2089-2096
***World Journal of Gastroenterology*** ISSN 1007-9327




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

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Received: December 13, 2008 Revised: February 19, 2009
Accepted: February 26, 2009
Published online: May 7, 2009

## 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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Schimanski CC, Frerichs K, Rahman F, Berger M, Lang H, Galle PR, Moehler M, Gockel I. High *miR-196a* levels promote the oncogenic phenotype of colorectal cancer cells. *World J Gastroenterol* 2009; 15(17): 2089-2096 Available from: URL: http://www.wjgnet.com/1007-9327/15/2089.asp DOI: http://dx.doi.org/10.3748/wjg.15.2089

## 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

$3 \times 10^5$ 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

$6 \times 10^3$ 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, $4 \times 10^4$ 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, $4 \times 10^4$ 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

$3 \times 10^5$ 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. $4 \times 10^4$ 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*:

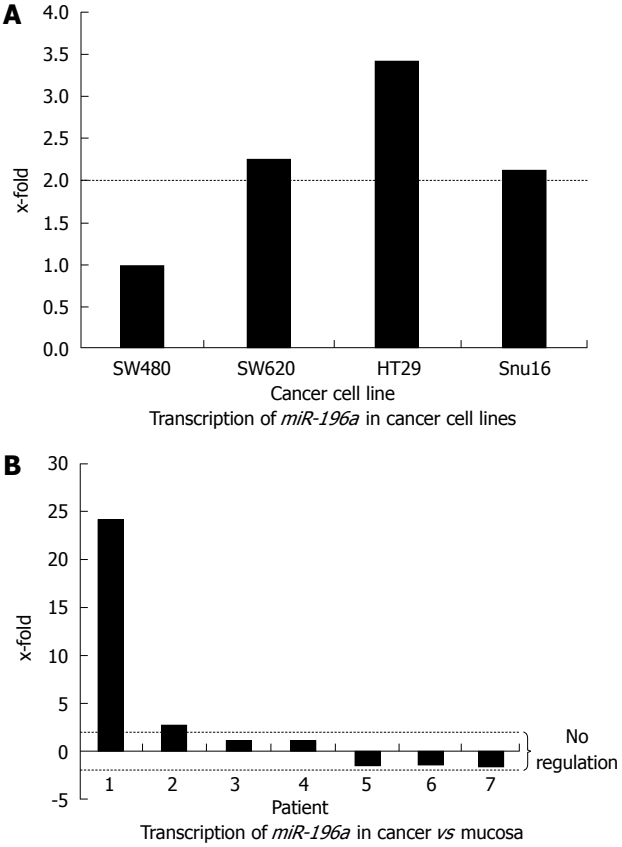


**Figure 1 Transcription levels of *miR-196a* in cancer cell lines and human CRC.** A: Cancer cell lines SW620, HT29 and Snu16 reveal increased *miR-196a* levels as compared to the primary colon cancer cell line SW480; B: *miR-196a* transcription is up-regulated in two of seven cancer samples in comparison 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 $\chi^2$ 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).

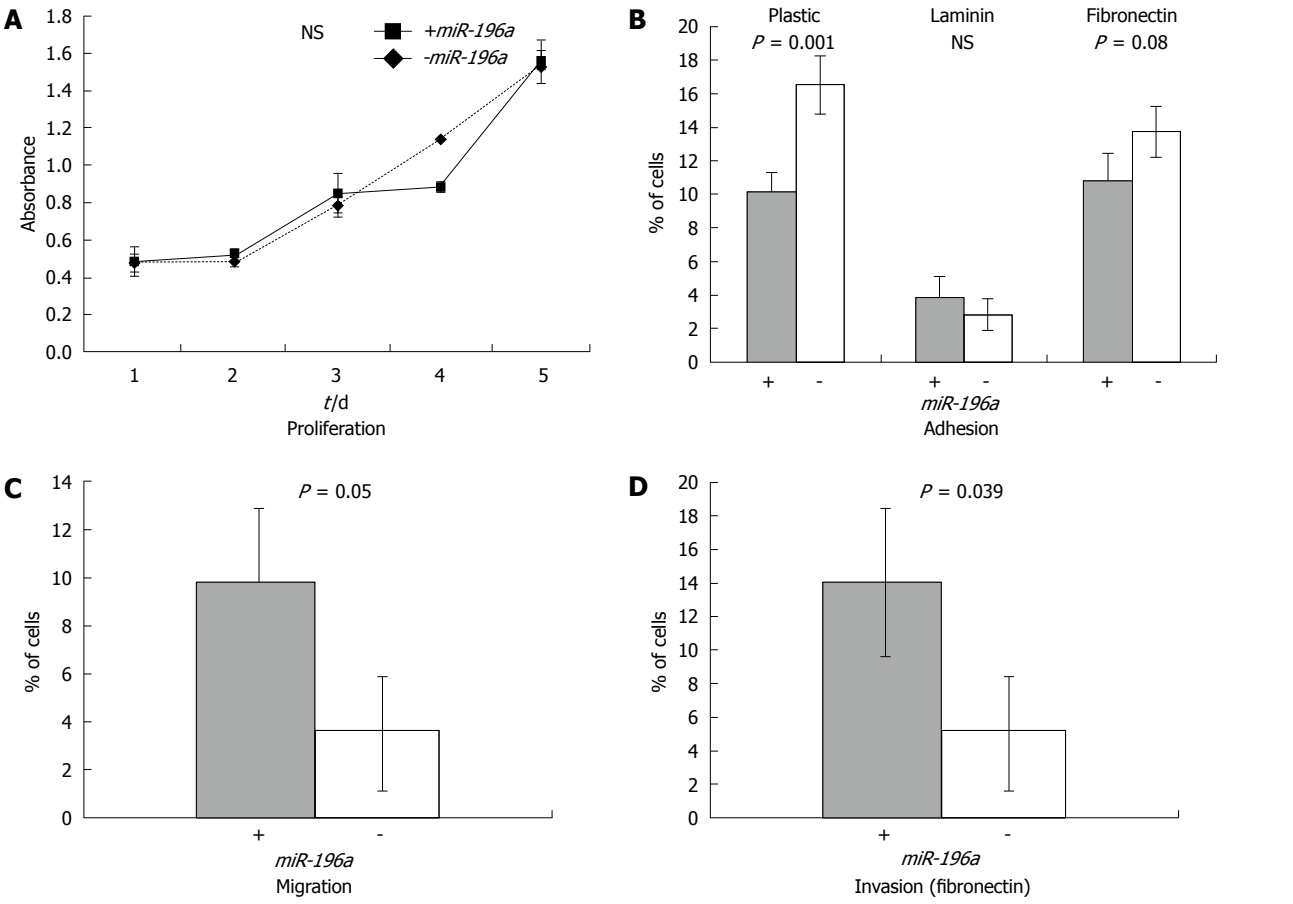


**Figure 2** ***In vitro*** **effect of** ***miR-196a*** **in human CRC.** Transient *miR-196a* transfection significantly decreases adhesion, increases migration and invasion but does not impact on proliferation or apoptosis of SW480 colon cancer cells.

### *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

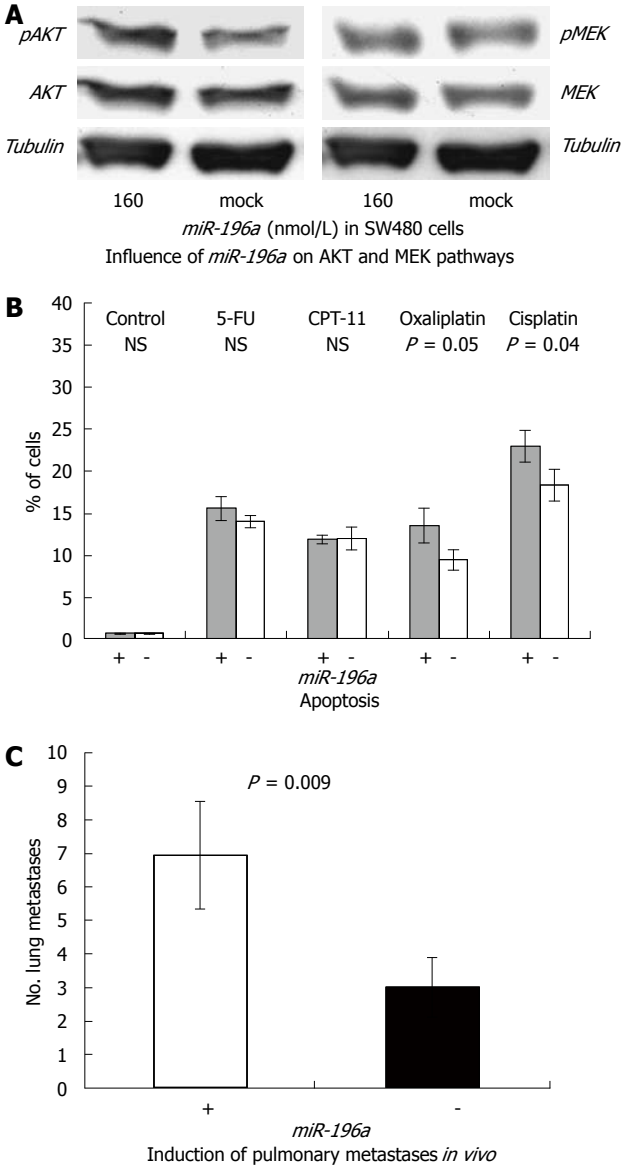


**Figure 3 Impact of *miR-196a* on cellular signaling, *in vitro* chemosensitivity and *in vivo* induction of lung metastases.** A: *miR-196a* transfection activates the AKT pathway but does not impact on the MEK pathway; B: *miR-196a* transfection significantly increases chemosensitivity towards oxaliplatin 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.

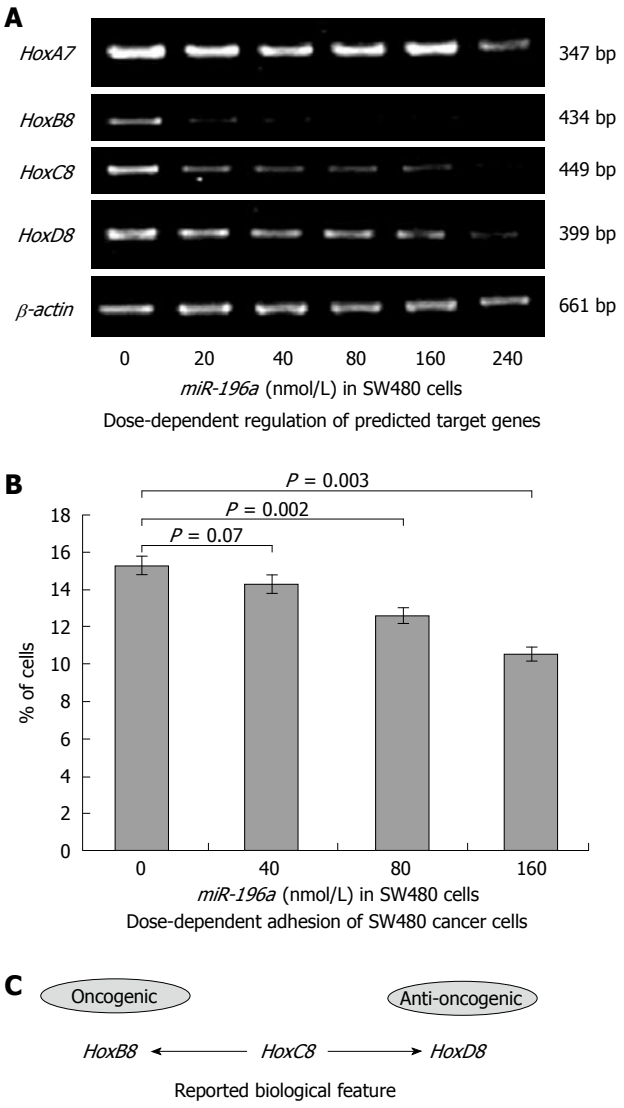


**Figure 4 Dose-dependence of *miR-196a* promoted effects.** A: *miR-196a* decreases *HoxA7*, *HoxB8*, *HoxC8* and *HoxD8* mRNA levels with a dose-dependent and gene-specific character; B: *miR-196a* inhibits cancer cell adhesion to plastic covers in a dose-dependent manner; C: Biological features of *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.

## REFERENCES


1 **Weir HK**, Thun MJ, Hankey BF, Ries LA, Howe HL, Wingo PA, Jemal A, Ward E, Anderson RN, Edwards BK. Annual report to the nation on the status of cancer, 1975-2000, featuring the uses of surveillance data for cancer prevention and control. *J Natl Cancer Inst* 2003; **95**: 1276-1299

2 **Greenlee RT**, Murray T, Bolden S, Wingo PA. Cancer statistics, 2000. *CA Cancer J Clin* 2000; **50**: 7-33

3 **August DA**, Ottow RT, Sugarbaker PH. Clinical perspective of human colorectal cancer metastasis. *Cancer Metastasis Rev* 1984; **3**: 303-324

4 **Fearon ER**, Vogelstein B. A genetic model for colorectal tumorigenesis. *Cell* 1990; **61**: 759-767

5 **Cho KR**, Vogelstein B. Genetic alterations in the adenoma--carcinoma sequence. *Cancer* 1992; **70**: 1727-1731

6 **Vogelstein B**, Kinzler KW. The multistep nature of cancer. *Trends Genet* 1993; **9**: 138-141

7 **Smith G**, Carey FA, Beattie J, Wilkie MJ, Lightfoot TJ, Coxhead J, Garner RC, Steele RJ, Wolf CR. Mutations in APC, Kirsten-ras, and p53--alternative genetic pathways to colorectal cancer. *Proc Natl Acad Sci USA* 2002; **99**: 9433-9438

8 **Ambros V**. microRNAs: tiny regulators with great potential. *Cell* 2001; **107**: 823-826

9 **Bartel DP**. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 2004; **116**: 281-297

10 **Carrington JC**, Ambros V. Role of microRNAs in plant and animal development. *Science* 2003; **301**: 336-338

11 **de Moor CH**, Meijer H, Lissenden S. Mechanisms of translational control by the 3' UTR in development and differentiation. *Semin Cell Dev Biol* 2005; **16**: 49-58
12 **Stark A**, Brennecke J, Bushati N, Russell RB, Cohen SM. Animal MicroRNAs confer robustness to gene expression and have a significant impact on 3'UTR evolution. *Cell* 2005; **123**: 1133-1146
13 **Lai EC**. Micro RNAs are complementary to 3' UTR sequence motifs that mediate negative post-transcriptional regulation. *Nat Genet* 2002; **30**: 363-364
14 **Calin GA**, Sevignani C, Dumitru CD, Hyslop T, Noch E, Yendamuri S, Shimizu M, Rattan S, Bullrich F, Negrini M, Croce CM. Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. *Proc Natl Acad Sci USA* 2004; **101**: 2999-3004
15 **Wynter CV**. The dialectics of cancer: A theory of the initiation and development of cancer through errors in RNAi. *Med Hypotheses* 2006; **66**: 612-635
16 **Takamizawa J**, Konishi H, Yanagisawa K, Tomida S, Osada H, Endoh H, Harano T, Yatabe Y, Nagino M, Nimura Y, Mitsudomi T, Takahashi T. Reduced expression of the let-7 microRNAs in human lung cancers in association with shortened postoperative survival. *Cancer Res* 2004; **64**: 3753-3756
17 **Akao Y**, Nakagawa Y, Naoe T. let-7 microRNA functions as a potential growth suppressor in human colon cancer cells. *Biol Pharm Bull* 2006; **29**: 903-906
18 **Jay C**, Nemunaitis J, Chen P, Fulgham P, Tong AW. miRNA profiling for diagnosis and prognosis of human cancer. *DNA Cell Biol* 2007; **26**: 293-300
19 **Johnson SM**, Grosshans H, Shingara J, Byrom M, Jarvis R, Cheng A, Labourier E, Reinert KL, Brown D, Slack FJ. RAS is regulated by the let-7 microRNA family. *Cell* 2005; **120**: 635-647
20 **Hayashita Y**, Osada H, Tatematsu Y, Yamada H, Yanagisawa K, Tomida S, Yatabe Y, Kawahara K, Sekido Y, Takahashi T. A polycistronic microRNA cluster, miR-17-92, is overexpressed in human lung cancers and enhances cell proliferation. *Cancer Res* 2005; **65**: 9628-9632
21 **Lewis BP**, Shih IH, Jones-Rhoades MW, Bartel DP, Burge CB. Prediction of mammalian microRNA targets. *Cell* 2003; **115**: 787-798
22 **Yekta S**, Shih IH, Bartel DP. MicroRNA-directed cleavage of HOXB8 mRNA. *Science* 2004; **304**: 594-596
23 **Hornstein E**, Mansfield JH, Yekta S, Hu JK, Harfe BD, McManus MT, Baskerville S, Bartel DP, Tabin CJ. The microRNA miR-196 acts upstream of Hoxb8 and Shh in limb development. *Nature* 2005; **438**: 671-674
24 **Bloomston M**, Frankel WL, Petrocca F, Volinia S, Alder H, Hagan JP, Liu CG, Bhatt D, Taccioli C, Croce CM. MicroRNA expression patterns to differentiate pancreatic adenocarcinoma from normal pancreas and chronic pancreatitis. *JAMA* 2007; **297**: 1901-1908
25 **Schetter AJ**, Leung SY, Sohn JJ, Zanetti KA, Bowman ED, Yanaihara N, Yuen ST, Chan TL, Kwong DL, Au GK, Liu CG, Calin GA, Croce CM, Harris CC. MicroRNA expression profiles associated with prognosis and therapeutic outcome in colon adenocarcinoma. *JAMA* 2008; **299**: 425-436
26 **Vider BZ**, Zimber A, Chastre E, Gespach C, Halperin M, Mashiah P, Yaniv A, Gazit A. Deregulated expression of homeobox-containing genes, HOXB6, B8, C8, C9, and Cdx-1, in human colon cancer cell lines. *Biochem Biophys Res Commun* 2000; **272**: 513-518
27 **Knoepfler PS**, Sykes DB, Pasillas M, Kamps MP. HoxB8 requires its Pbx-interaction motif to block differentiation of primary myeloid progenitors and of most cell line models of myeloid differentiation. *Oncogene* 2001; **20**: 5440-5448
28 **Perkins AC**, Cory S. Conditional immortalization of mouse myelomonocytic, megakaryocytic and mast cell progenitors by the Hox-2.4 homeobox gene. *EMBO J* 1993; **12**: 3835-3846
29 **Manohar CF**, Salwen HR, Furtado MR, Cohn SL. Up-regulation of HOXC6, HOXD1, and HOXD8 homeobox gene expression in human neuroblastoma cells following chemical induction of differentiation. *Tumour Biol* 1996; **17**: 34-47
30 **Nadauld LD**, Sandoval IT, Chidester S, Yost HJ, Jones DA. Adenomatous polyposis coli control of retinoic acid biosynthesis is critical for zebrafish intestinal development and differentiation. *J Biol Chem* 2004; **279**: 51581-51589
31 **Waltregny D**, Alami Y, Clausse N, de Leval J, Castronovo V. Overexpression of the homeobox gene HOXC8 in human prostate cancer correlates with loss of tumor differentiation. *Prostate* 2002; **50**: 162-169
32 **Miller GJ**, Miller HL, van Bokhoven A, Lambert JR, Werahera PN, Schirripa O, Lucia MS, Nordeen SK. Aberrant HOXC expression accompanies the malignant phenotype in human prostate. *Cancer Res* 2003; **63**: 5879-5888


**S- Editor** Li LF **L- Editor** O'Neill M **E- Editor** Zheng XM

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.15.2097

*World J Gastroenterol* 2009 May 7; 15(17): 2097-2108
***World Journal of Gastroenterology*** ISSN 1007-9327




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

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Received: December 19, 2008 Revised: March 20, 2009
Accepted: March 27, 2009
Published online: May 7, 2009

## 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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Renga B, Mencarelli A, Migliorati M, Distrutti E, Fiorucci S. Bile-acid-activated farnesoid X receptor regulates hydrogen sulfide production and hepatic microcirculation. *World J Gastroenterol* 2009; 15(17): 2097-2108 Available from: URL: http://www.wjgnet.com/1007-9327/15/2097.asp DOI: http://dx.doi.org/10.3748/wjg.15.2097

## 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, $10 \times 10^5$ 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

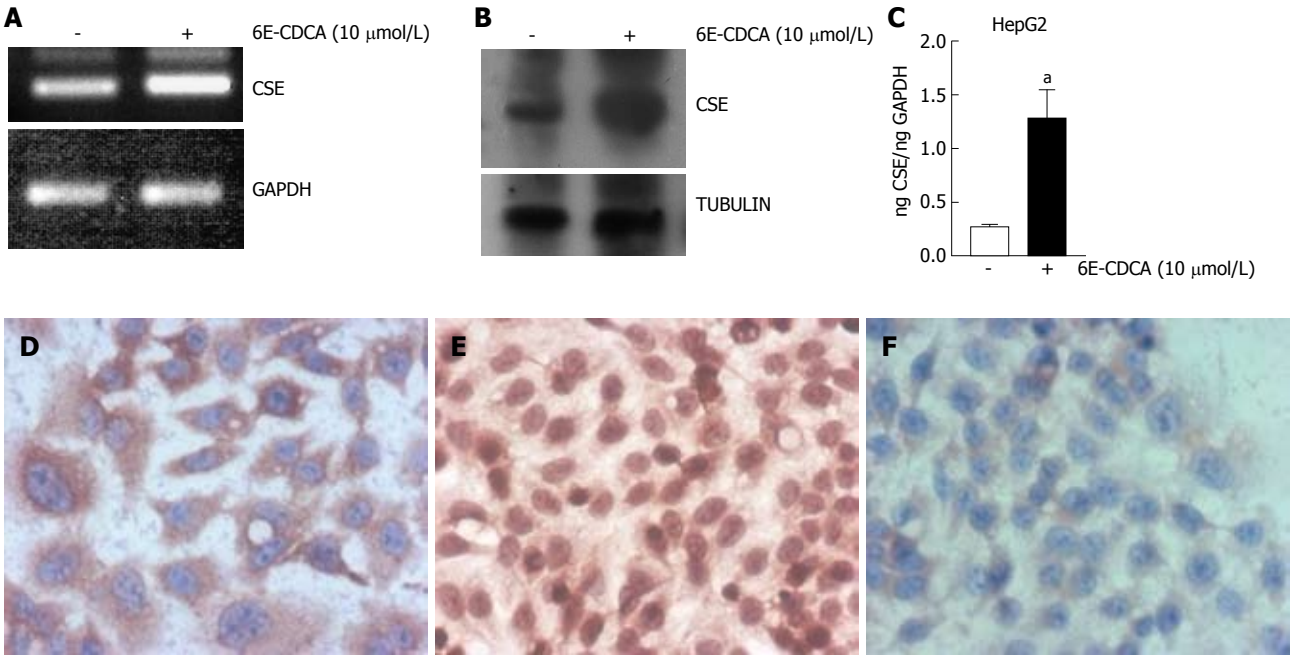


**Figure 1** ***CSE*** **gene expression is regulated by bile acids.** A, B: Qualitative and quantitative PCR showing the up-regulation of CSE mRNA in HepG2 cell line stimulated with FXR ligand 6E-CDCA (10 μmol/L) for 18 h. Data are shown as mean ± SD of three experiments. $^{a}P < 0.05$ *versus* not 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

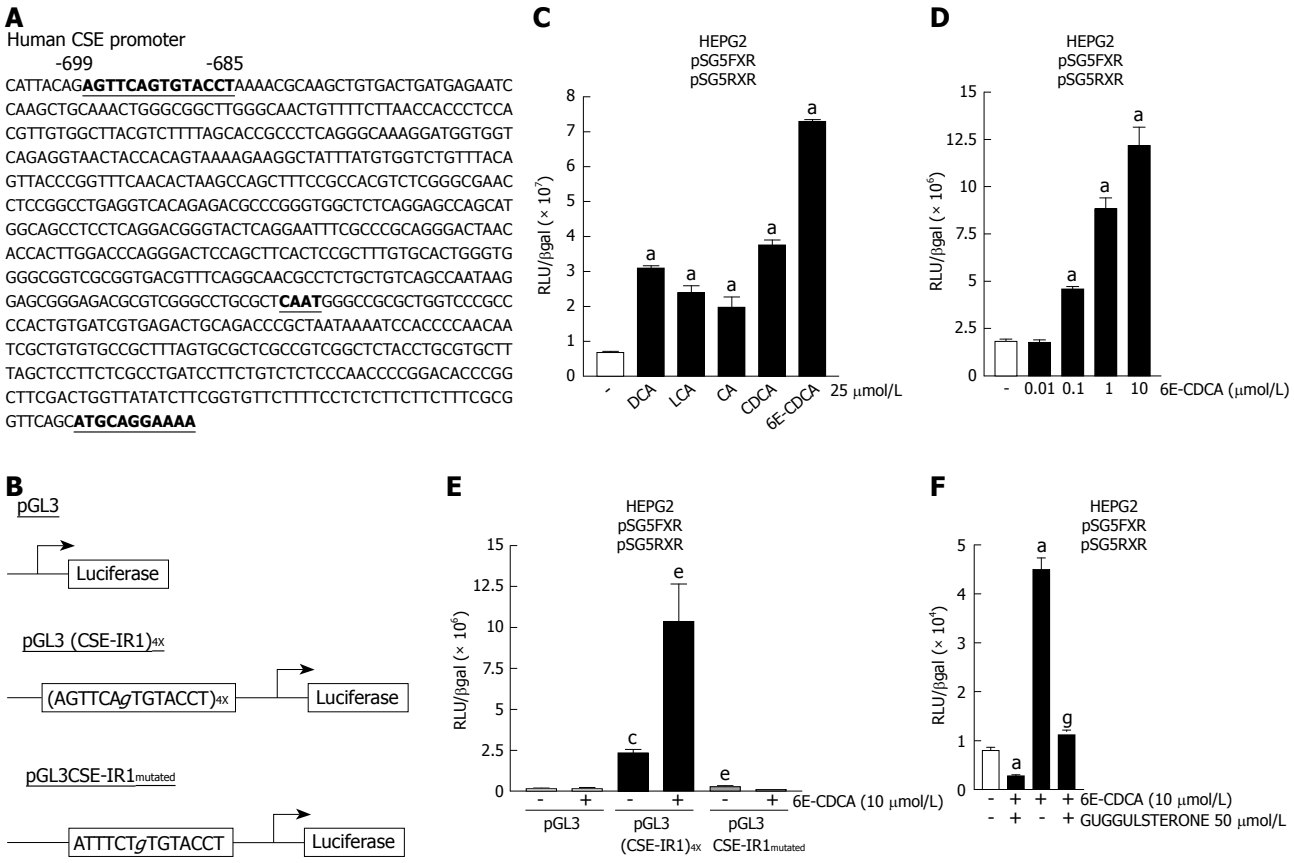


**Figure 2 An FXR responsive element is expressed in the CSE promoter.** A: Analysis of the promoter of the human *CSE* gene, showing a putative IR-1 site at -699/-685 base pairs upstream of the transcriptional start site ATG; B: Schematic representation of reporter constructs containing four CSE-IR1 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

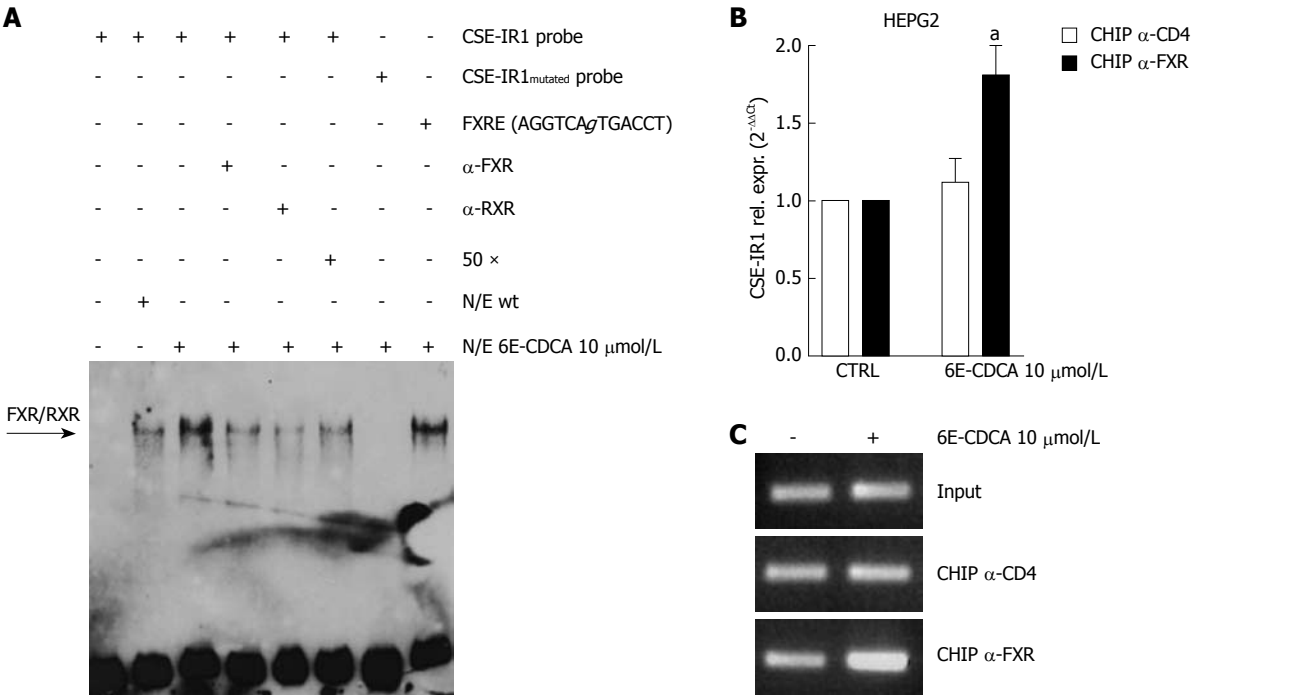


**Figure 3 Activation of FXR regulates CSE expression.** A: FXR/RXR bind to CSE-IR1 of the *CSE* gene. EMSAs were performed to analyze binding of FXR/RXR to the putative IR1 sequence in the CSE gene. CSE-IR1, CSE-IR1$_{mutated}$ and FXRE-IR1 probes, biotin-labeled, were used in this experiment. CSERE-IR1 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.

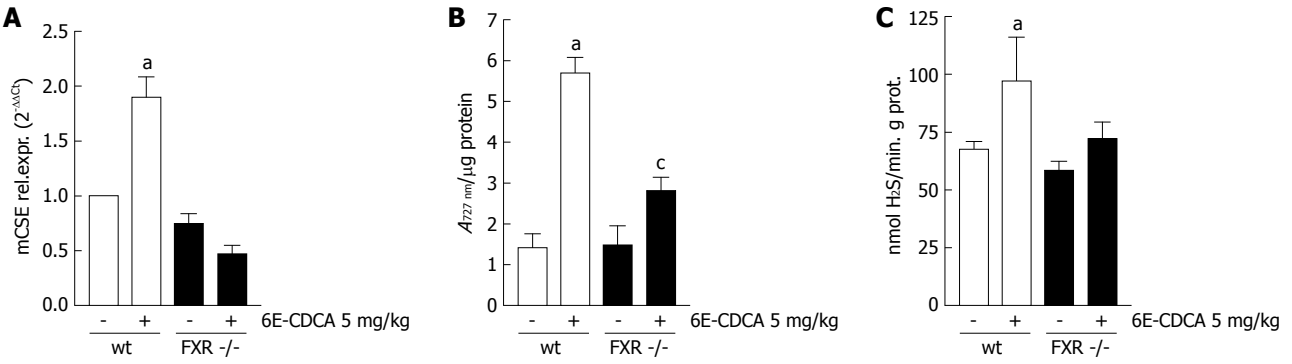


**Figure 4 CSE expression/activity is regulated with an FXR ligand *in vivo*.** A: FXR +/+ and FXR -/- mice were treated for 3 d with vehicle or with 6E-CDCA 5 mg/kg body weight. Total RNA from liver of FXR +/+ and FXR -/- mice was subjected to real-time PCR quantification of *CSE* gene expression. $^{a}P < 0.05$ *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 +/+

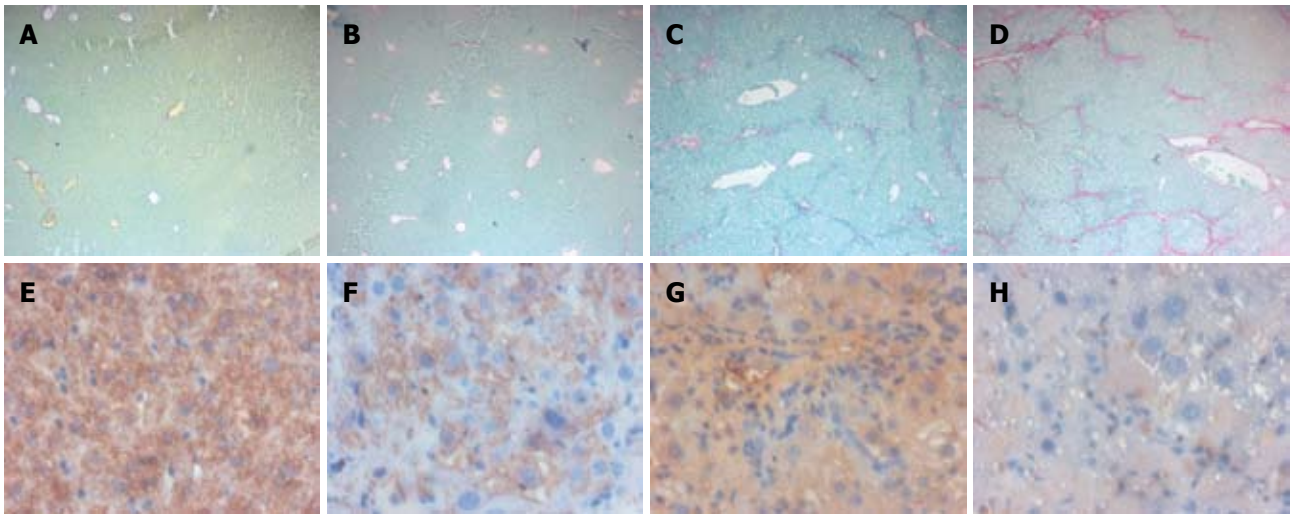


**Figure 5 FXR loss of function sensitizes mice to $CCl_4$-induced liver fibrosis.** A: Sirius red staining of liver section obtained from FXR +/+ mice; B: Sirius red staining of liver section obtained from FXR -/- mice; C: Sirius red staining of liver section obtained from FXR +/+ mice treated with $CCl_4$; 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 $\alpha$1-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.

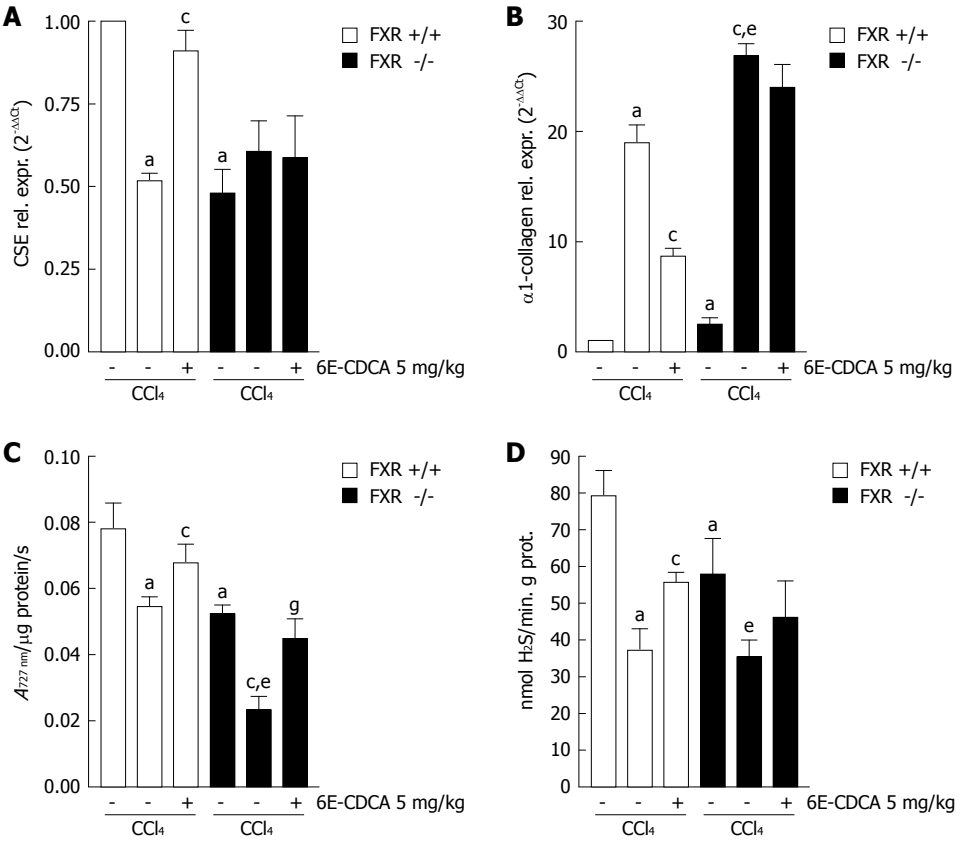


**Figure 6 FXR activation induces *CSE* gene expression and regulated CSE activity in the liver with cirrhosis.** FXR +/+ and FXR -/- mice were treated with $CCl_4$ and 6E-CDCA as described in the methods section. A and B: Quantitative real-time PCR of CSE mRNA and α1-collagen mRNA from FXR +/+ and FXR -/- 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

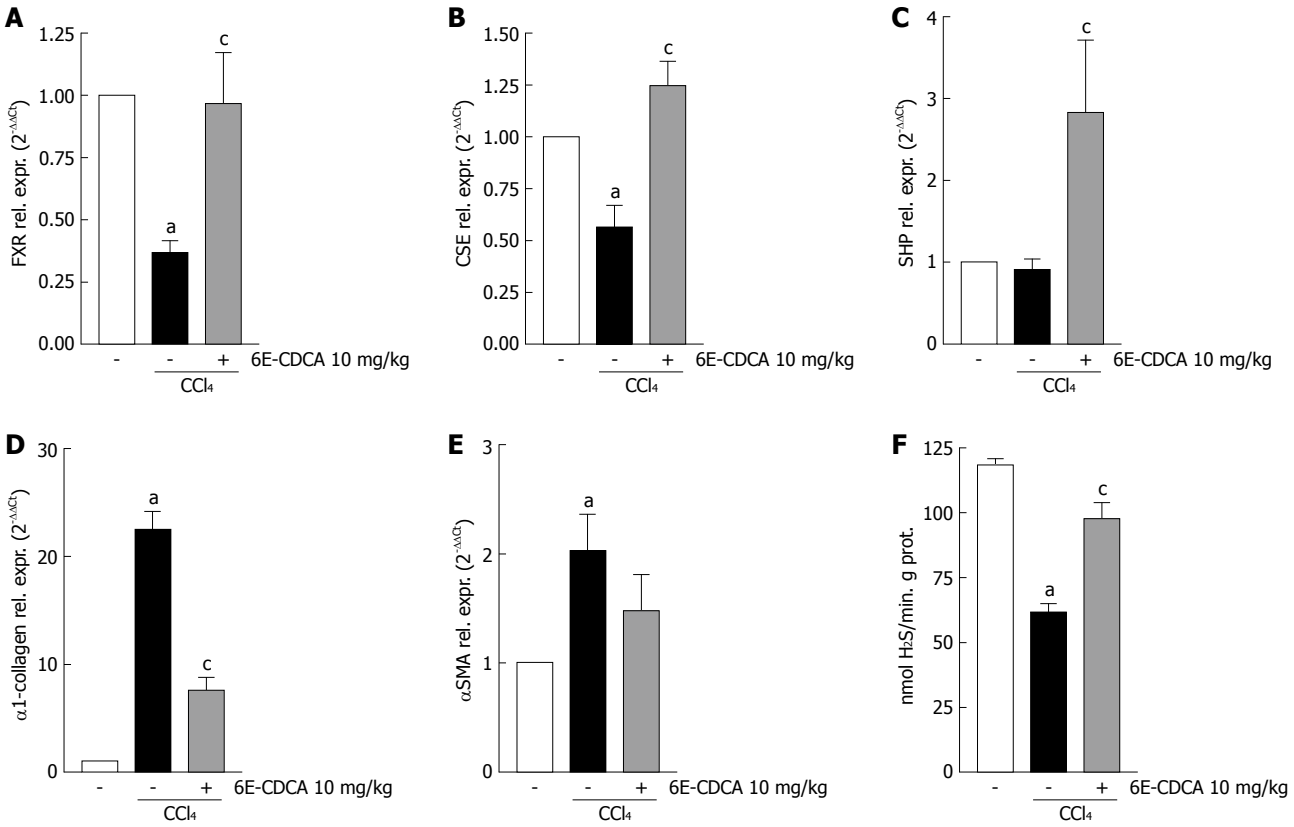


**Figure 7 FXR activation induces both CSE mRNA expression and activity in rats with liver cirrhosis.** Quantitative real-time PCR of (A) FXR mRNA, (B) CSE mRNA, (C) SHP mRNA, (D) α1-collagen mRNA, (E) αSMA mRNA from rats liver homogenates and (F) Rat liver CSE activity. Data are mean ± SD of six mice.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

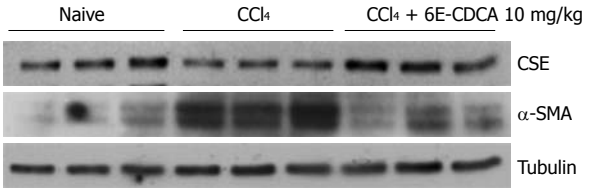


**Figure 8 FXR activation induces CSE protein expression and reduces αSMA protein level in rat's liver with cirrhosis.** Western blotting analysis of CSE, αSMA and tubulin on liver homogenates. From left to right: Lanes 1-3, liver samples from control rats; Lanes 4-6, liver samples from rats administered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

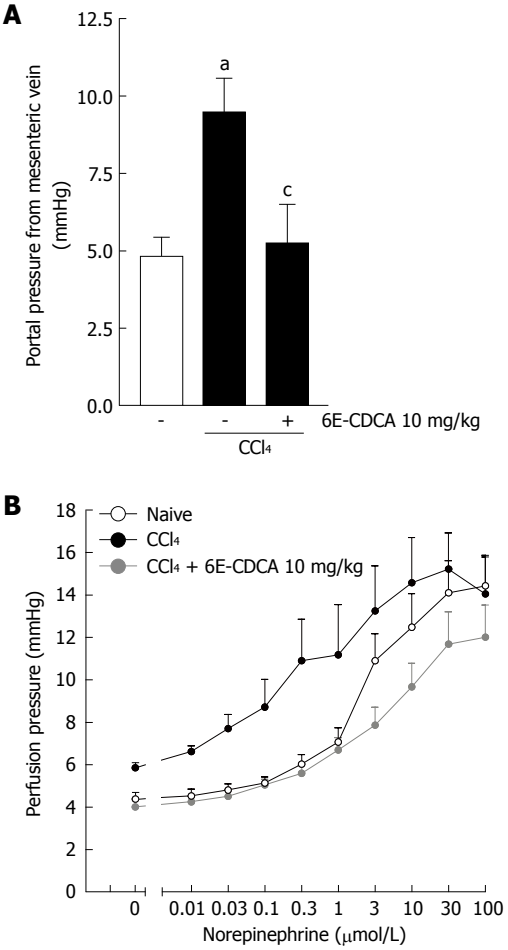


**Figure 9 FXR activation reduces portal pressure in rat liver with cirrhosis.** Data are mean ± SD of six mice. A: Basal portal pressure from mesenteric vein. $^{a}P < 0.05$ *versus* control rats. $^{c}P < 0.05$ *versus* $CCl_4$ rats; B: Effects of 6E-CDCA on pre-contracted rat liver with cirrhosis.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

## REFERENCES


1 **Ishii I**, Akahoshi N, Yu XN, Kobayashi Y, Namekata K, Komaki G, Kimura H. Murine cystathionine gamma-lyase: complete cDNA and genomic sequences, promoter activity, tissue distribution and developmental expression. *Biochem J* 2004; **381**: 113-123

2 **Yamanishi T**, Tuboi S. The mechanism of the L-cystine cleavage reaction catalyzed by rat liver gamma-cystathionase. *J Biochem* 1981; **89**: 1913-1921

3 **Stipanuk MH**. Sulfur amino acid metabolism: pathways for production and removal of homocysteine and cysteine. *Annu Rev Nutr* 2004; **24**: 539-577

4 **Kim SK**, Choi KH, Kim YC. Effect of acute betaine administration on hepatic metabolism of S-amino acids in rats and mice. *Biochem Pharmacol* 2003; **65**: 1565-1574

5 **Rao AM**, Drake MR, Stipanuk MH. Role of the transsulfuration pathway and of gamma-cystathionase activity in the formation of cysteine and sulfate from methionine in rat hepatocytes. *J Nutr* 1990; **120**: 837-845

6 **Triguero A**, Barber T, García C, Puertes IR, Sastre J, Viña JR. Liver intracellular L-cysteine concentration is maintained after inhibition of the trans-sulfuration pathway by propargylglycine in rats. *Br J Nutr* 1997; **78**: 823-831

7 **Stipanuk MH**, Dominy JE Jr, Lee JI, Coloso RM. Mammalian cysteine metabolism: new insights into regulation of cysteine metabolism. *J Nutr* 2006; **136**: 1652S-1659S

8 **Drake MR**, De La Rosa J, Stipanuk MH. Metabolism of cysteine in rat hepatocytes. Evidence for cysteinesulphinate-independent pathways. *Biochem J* 1987; **244**: 279-286

9 **Hosoki R**, Matsuki N, Kimura H. The possible role of hydrogen sulfide as an endogenous smooth muscle relaxant in synergy with nitric oxide. *Biochem Biophys Res Commun* 1997; **237**: 527-531

10 **Teague B**, Asiedu S, Moore PK. The smooth muscle relaxant effect of hydrogen sulphide in vitro: evidence for a physiological role to control intestinal contractility. *Br J Pharmacol* 2002; **137**: 139-145

11 **Cheng Y**, Ndisang JF, Tang G, Cao K, Wang R. Hydrogen sulfide-induced relaxation of resistance mesenteric artery beds of rats. *Am J Physiol Heart Circ Physiol* 2004; **287**: H2316-H2323

12 **Zhao W**, Zhang J, Lu Y, Wang R. The vasorelaxant effect of H(2)S as a novel endogenous gaseous K(ATP) channel opener. *EMBO J* 2001; **20**: 6008-6016

13 **Fiorucci S**, Antonelli E, Distrutti E, Rizzo G, Mencarelli A, Orlandi S, Zanardo R, Renga B, Di Sante M, Morelli A, Cirino G, Wallace JL. Inhibition of hydrogen sulfide generation contributes to gastric injury caused by anti-inflammatory nonsteroidal drugs. *Gastroenterology* 2005; **129**: 1210-1224

14 **Zhong G**, Chen F, Cheng Y, Tang C, Du J. The role of hydrogen sulfide generation in the pathogenesis of hypertension in rats induced by inhibition of nitric oxide synthase. *J Hypertens* 2003; **21**: 1879-1885

15 **Bellentani S**, Pecorari M, Cordoma P, Marchegiano P, Manenti F, Bosisio E, De Fabiani E, Galli G. Taurine increases bile acid pool size and reduces bile saturation index in the hamster. *J Lipid Res* 1987; **28**: 1021-1027

16 **Murakami S**, Kondo Y, Toda Y, Kitajima H, Kameo K, Sakono M, Fukuda N. Effect of taurine on cholesterol metabolism in hamsters: up-regulation of low density lipoprotein (LDL) receptor by taurine. *Life Sci* 2002; **70**: 2355-2366

17 **Fiorucci S**, Antonelli E, Mencarelli A, Orlandi S, Renga B, Rizzo G, Distrutti E, Shah V, Morelli A. The third gas: H2S regulates perfusion pressure in both the isolated and perfused normal rat liver and in cirrhosis. *Hepatology* 2005; **42**: 539-548

18 **Distrutti E**, Mencarelli A, Santucci L, Renga B, Orlandi S, Donini A, Shah V, Fiorucci S. The methionine connection: homocysteine and hydrogen sulfide exert opposite effects on hepatic microcirculation in rats. *Hepatology* 2008; **47**: 659-667

19 **Forman BM**, Goode E, Chen J, Oro AE, Bradley DJ, Perlmann T, Noonan DJ, Burka LT, McMorris T, Lamph WW, Evans RM, Weinberger C. Identification of a nuclear receptor that is activated by farnesol metabolites. *Cell* 1995; **81**: 687-693

20 **Seol W**, Choi HS, Moore DD. Isolation of proteins that interact specifically with the retinoid X receptor: two novel orphan receptors. *Mol Endocrinol* 1995; **9**: 72-85

21 **Pellicciari R**, Costantino G, Fiorucci S. Farnesoid X receptor: from structure to potential clinical applications. *J Med Chem* 2005; **48**: 5383-5403

22 **Edwards PA**, Kast HR, Anisfeld AM. BAREing it all: the adoption of LXR and FXR and their roles in lipid homeostasis. *J Lipid Res* 2002; **43**: 2-12

23 **Nishi N**, Tanabe H, Oya H, Urushihara M, Miyanaka H, Wada F. Identification of probasin-related antigen as cystathionine gamma-lyase by molecular cloning. *J Biol Chem* 1994; **269**: 1015-1019

24 **Ogasawara Y**, Ishii K, Tanabe S. Enzymatic assay of gamma-cystathionase activity using pyruvate oxidase-peroxidase sequential reaction. *J Biochem Biophys Methods* 2002; **51**: 139-150

25 **Sinal CJ**, Tohkin M, Miyata M, Ward JM, Lambert G, Gonzalez FJ. Targeted disruption of the nuclear receptor FXR/BAR impairs bile acid and lipid homeostasis. *Cell* 2000; **102**: 731-744

26 **Fiorucci S**, Antonelli E, Morelli O, Mencarelli A, Casini A, Mello T, Palazzetti B, Tallet D, del Soldato P, Morelli A. NCX-1000, a NO-releasing derivative of ursodeoxycholic acid, selectively delivers NO to the liver and protects against development of portal hypertension. *Proc Natl Acad Sci USA* 2001; **98**: 8897-8902

27 **Grossman HJ**, Grossman VL, Bhathal PS. Hemodynamic characteristics of the intrahepatic portal vascular bed over an extended flow range: a study in the isolated perfused rat liver. *Hepatology* 1995; **21**: 162-168

28 **Shah V**. Cellular and molecular basis of portal hypertension. *Clin Liver Dis* 2001; **5**: 629-644

29 **Shah V**. Molecular mechanisms of increased intrahepatic resistance in portal hypertension. *J Clin Gastroenterol* 2007; **41** Suppl 3: S259-S261

30 **Bosch J**, Pizcueta P, Feu F, Fernàndez M, Garcìa-Pagan JC. Pathophysiology of portal hypertension. *Gastroenterol Clin North Am* 1992; **21**: 1-14

31 **Gonzàlez-Abraldes J**, Garcìa-Pagan JC, Bosch J. Nitric oxide and portal hypertension. *Metab Brain Dis* 2002; **17**: 311-324

32 **Moreau R**, Lebrec D. Molecular and structural basis of portal hypertension. *Clin Liver Dis* 2006; **10**: 445-457, vii

33 **Bosch J**, Garcìa-Pagan JC. Complications of cirrhosis. I. Portal hypertension. *J Hepatol* 2000; **32**: 141-156


**S- Editor** Li LF **L- Editor** Stewart GJ **E- Editor** Lin YP

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.15.2109

*World J Gastroenterol* 2009 May 7; 15(17): 2109-2115
***World Journal of Gastroenterology*** ISSN 1007-9327




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

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Received: January 23, 2009 Revised: March 24, 2009
Accepted: March 31, 2009
Published online: May 7, 2009

## 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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Yang MF, Xie J, Gu XY, Zhang XH, Davey AK, Zhang SJ, Wang JP, Zhu RM. Involvement of 90-kuD ribosomal S6 kinase in collagen type Ⅰ expression in rat hepatic fibrosis. *World J Gastroenterol* 2009; 15(17): 2109-2115 Available from: URL: http://www.wjgnet.com/1007-9327/15/2109.asp DOI: http://dx.doi.org/10.3748/wjg.15.2109

## 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 $\Delta C_t$, 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 ($4 \times 10^6$), 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.

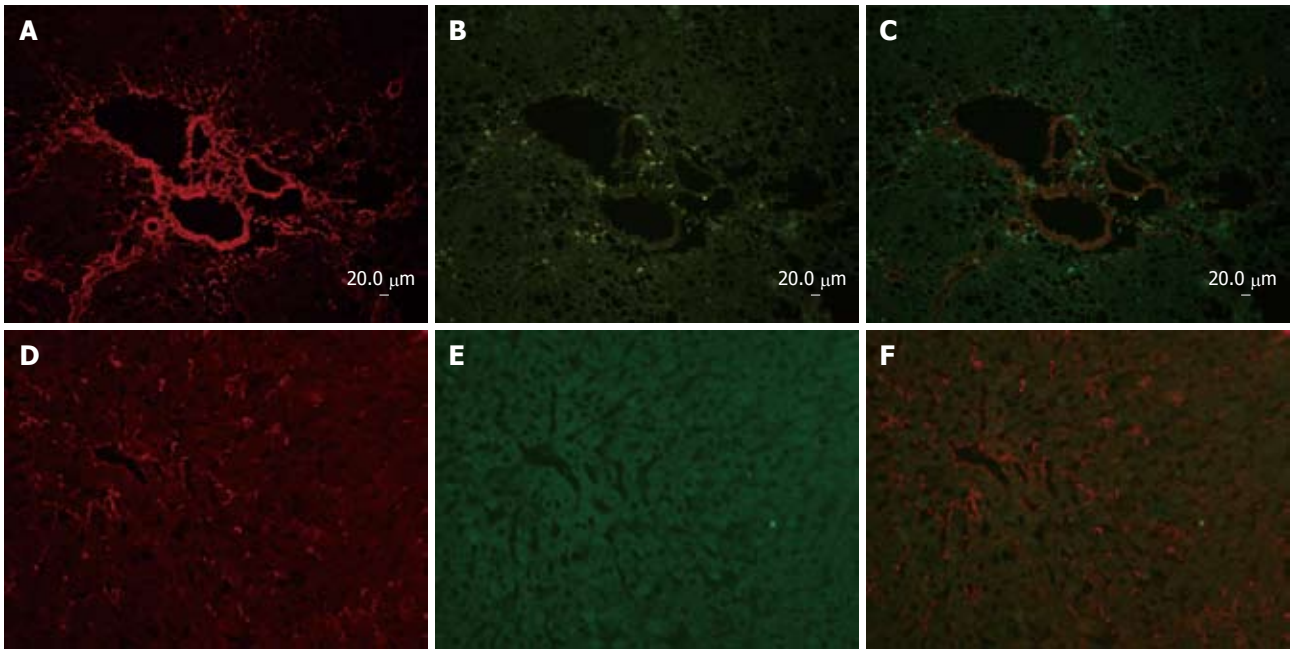


**Figure 1 Co-immunofluoresence of p90RSK and collagen type Ⅰ in fibrotic liver and normal liver.** Sections (A-C) are from rat liver with intraperitoneal injection of DMN for 3 wk, and sections (D-F) are from normal livers as control. Sections of fibrotic liver mostly demonstrate that collagen type 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.

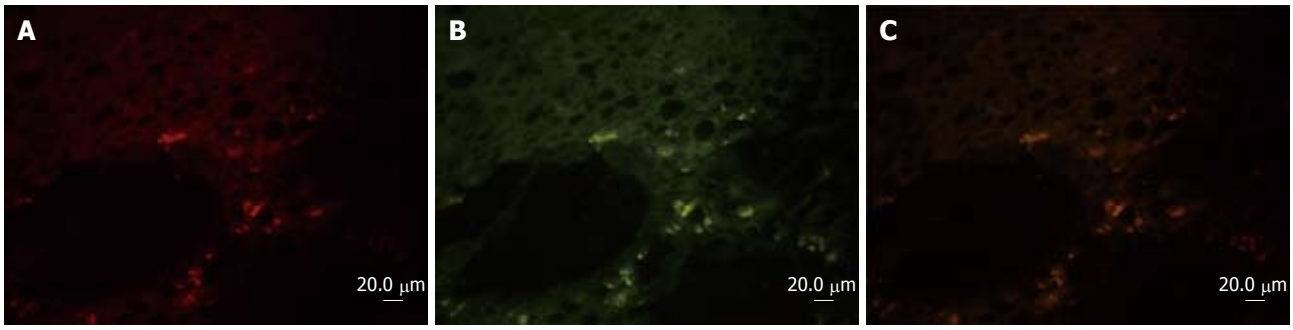


**Figure 2 Cellular localization of p90RSK in fibrotic liver and co-immunofluoresence and confocal microscopy of p90RSK and αSMA in fibrotic liver.** A: α-SMA (rhodamine)-positive cell represent the activated HSC, which deposited in interstitium; B: P90RSK (FITC)-positive cell were also present in interstitium;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

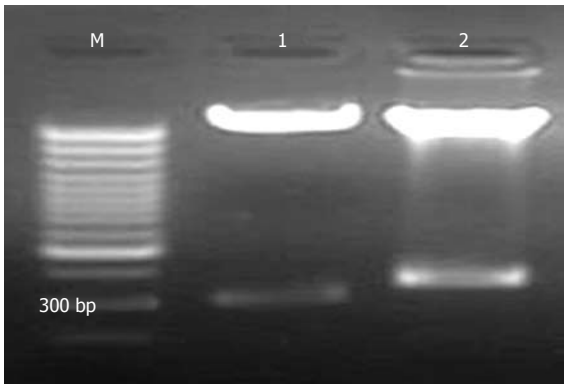


**Figure 3 Agarose gel electrophoresis of restriction enzyme digestion of pAV-siRSK.** M: 1 kb marker; 1: Restriction enzyme digestion product of pAVU6+27 was about 300 bp; 2: Restriction enzyme digestion product of pAV-siRSK. The siRNA of p90RSK was designed of 52 bp.

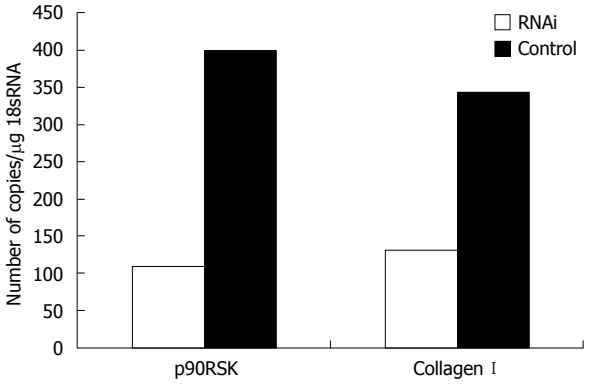


**Figure 4 RT-PCR assessment of p90RSK and collagen type I in HSC-T6 transfected with or without pAVU6-siRSK.** Quantification of p90RSK and collagen type I normalized to 18S RNA in HSC-T6 cells transfected with pAVU6-siRSK decreased 72.6% and 61.8%, respectively, compared with control ($P < 0.01$).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

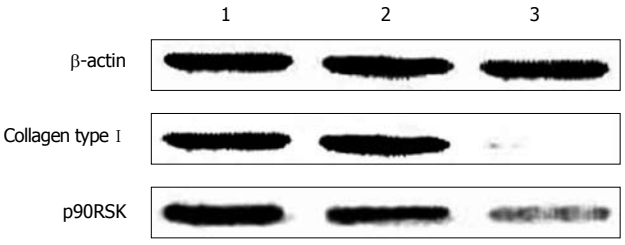


**Figure 5 Western blotting analysis of p90RSK, collagen type I in HSC-T6 cells transfected with or without pAVU6-siRSK.** β-actin provided as an inner control. 1: Normal HSC-T6 cells; 2: HSC-T6 transfected with empty plasmid; 3: HSC-T6 transfected with pAVU6-siRSK. With p90RSK siRNA, the expression 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

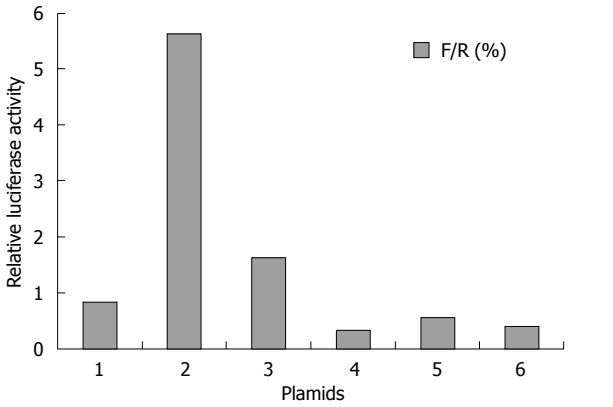


**Figure 6 Effect of p90RSK on collagen type I promoter activity.** HSCs transfected with the pAVU6-siRSK (bar 3, to decrease p90RSK expression), pMT2·RSK1 (bar 5, to increase p90RSK expression) showed no alteration of collagen type I promoter activity compared to control cells sham-transfected with 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.

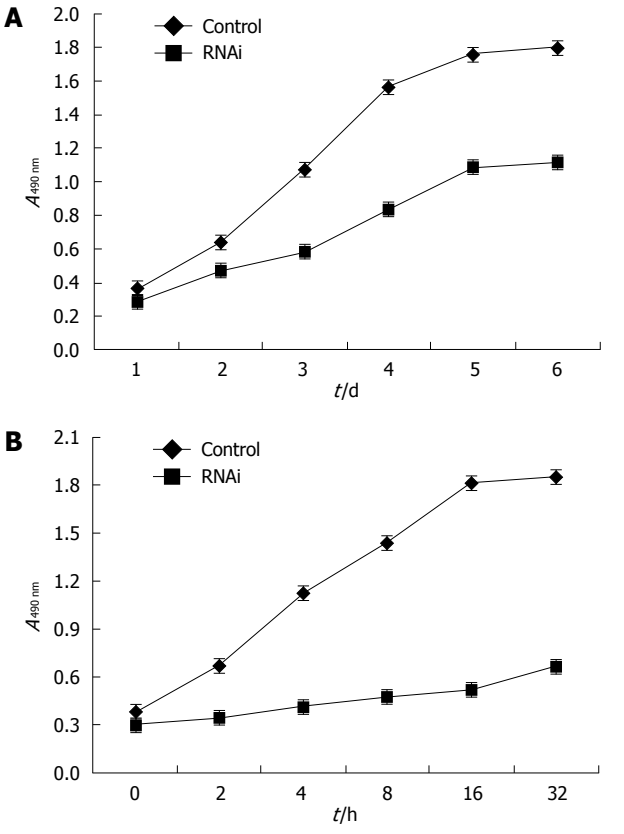


**Figure 7 Effect of p90RSK siRNA on the proliferation of HSCs.** A: p90RSK siRNA inhibited the proliferation of HSC-T6 cells. Cell growth curves of the recombinant cells with or without p90RSK siRNA were analyzed by MTS conversion; B: p90RSK siRNA abolished the effect of PDGF-BB on the proliferation 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.

## ACKNOWLEDGMENTS

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.

## REFERENCES


1 **Brenner DA**, Rippe RA, Rhodes K, Trotter JF, Breindl M. Fibrogenesis and type I collagen gene regulation. *J Lab Clin Med* 1994; **124**: 755-760
2 **Friedman SL**. Cytokines and fibrogenesis. *Semin Liver Dis* 1999; **19**: 129-140
3 **Friedman SL**. Molecular regulation of hepatic fibrosis, an integrated cellular response to tissue injury. *J Biol Chem* 2000; **275**: 2247-2250
4 **Geerts A**, Lazou JM, De Bleser P, Wisse E. Tissue distribution, quantitation and proliferation kinetics of fat-storing cells in carbon tetrachloride-injured rat liver. *Hepatology* 1991; **13**: 1193-1202
5 **Takahara T**, Kojima T, Miyabayashi C, Inoue K, Sasaki H, Muragaki Y, Ooshima A. Collagen production in fat-storing cells after carbon tetrachloride intoxication in the rat. Immunoelectron microscopic observation of type I, type III collagens, and prolyl hydroxylase. *Lab Invest* 1988; **59**: 509-521
6 **Yin MF**, Lian LH, Piao DM, Nan JX. Tetrandrine stimulates the apoptosis of hepatic stellate cells and ameliorates development of fibrosis in a thioacetamide rat model. *World J Gastroenterol* 2007; **13**: 1214-1220
7 **Bonni A**, Brunet A, West AE, Datta SR, Takasu MA, Greenberg ME. Cell survival promoted by the Ras-MAPK signaling pathway by transcription-dependent and -independent mechanisms. *Science* 1999; **286**: 1358-1362
8 **Marra F**, Arrighi MC, Fazi M, Caligiuri A, Pinzani M, Romanelli RG, Efsen E, Laffi G, Gentilini P. Extracellular signal-regulated kinase activation differentially regulates platelet-derived growth factor's actions in hepatic stellate cells, and is induced by in vivo liver injury in the rat. *Hepatology* 1999; **30**: 951-958
9 **Pinzani M**, Marra F. Cytokine receptors and signaling in hepatic stellate cells. *Semin Liver Dis* 2001; **21**: 397-416
10 **Anjum R**, Roux PP, Ballif BA, Gygi SP, Blenis J. The tumor suppressor DAP kinase is a target of RSK-mediated survival signaling. *Curr Biol* 2005; **15**: 1762-1767
11 **Butcher GQ**, Lee B, Hsieh F, Obrietan K. Light- and clock-dependent regulation of ribosomal S6 kinase activity in the suprachiasmatic nucleus. *Eur J Neurosci* 2004; **19**: 907-915
12 **Mori M**, Hara M, Tachibana K, Kishimoto T. p90Rsk is required for G1 phase arrest in unfertilized starfish eggs. *Development* 2006; **133**: 1823-1830
13 **Richards SA**, Dreisbach VC, Murphy LO, Blenis J. Characterization of regulatory events associated with membrane targeting of p90 ribosomal S6 kinase 1. *Mol Cell Biol* 2001; **21**: 7470-7480
14 **Frödin M**, Gammeltoft S. Role and regulation of 90 kDa ribosomal S6 kinase (RSK) in signal transduction. *Mol Cell Endocrinol* 1999; **151**: 65-77
15 **Qiang H**, Lin Y, Zhang X, Zeng X, Shi J, Chen YX, Yang MF, Han ZG, Xie WF. Differential expression genes analyzed by cDNA array in the regulation of rat hepatic fibrogenesis. *Liver Int* 2006; **26**: 1126-1137
16 **Haratake J**, Hisaoka M, Yamamoto O, Horie A. Morphological changes of hepatic microcirculation in experimental rat cirrhosis: a scanning electron microscopic study. *Hepatology* 1991; **13**: 952-956
17 **Mérienne K**, Jacquot S, Zeniou M, Pannetier S, Sassone-Corsi P, Hanauer A. Activation of RSK by UV-light: phosphorylation dynamics and involvement of the MAPK pathway. *Oncogene* 2000; **19**: 4221-4229
18 **Vogel S**, Piantedosi R, Frank J, Lalazar A, Rockey DC, Friedman SL, Blaner WS. An immortalized rat liver stellate cell line (HSC-T6): a new cell model for the study of retinoid metabolism in vitro. *J Lipid Res* 2000; **41**: 882-893
19 **Slack JL**, Parker MI, Robinson VR, Bornstein P. Regulation of collagen I gene expression by ras. *Mol Cell Biol* 1992; **12**: 4714-4723
20 **Iraburu MJ**, Domínguez-Rosales JA, Fontana L, Auster A, García-Trevijano ER, Covarrubias-Pinedo A, Rivas-Estilla AM, Greenwel P, Rojkind M. Tumor necrosis factor alpha down-regulates expression of the alpha1(I) collagen gene in rat hepatic stellate cells through a p20C/EBPbeta- and C/EBPdelta-dependent mechanism. *Hepatology* 2000; **31**: 1086-1093
21 **Inagaki Y**, Truter S, Greenwel P, Rojkind M, Unoura M, Kobayashi K, Ramirez F. Regulation of the alpha 2(I) collagen gene transcription in fat-storing cells derived from a cirrhotic liver. *Hepatology* 1995; **22**: 573-579
22 **Mak KM**, Leo MA, Lieber CS. Alcoholic liver injury in baboons: transformation of lipocytes to transitional cells. *Gastroenterology* 1984; **87**: 188-200
23 **Ramadori G**, Veit T, Schwögler S, Dienes HP, Knittel T, Rieder H, Meyer zum Büschenfelde KH. Expression of the gene of the alpha-smooth muscle-actin isoform in rat liver and in rat fat-storing (ITO) cells. *Virchows Arch B Cell Pathol Incl Mol Pathol* 1990; **59**: 349-357
24 **Kim Y**, Ratziu V, Choi SG, Lalazar A, Theiss G, Dang Q, Kim SJ, Friedman SL. Transcriptional activation of transforming growth factor beta1 and its receptors by the Kruppel-like factor Zf9/core promoter-binding protein and Sp1. Potential mechanisms for autocrine fibrogenesis in response to injury. *J Biol Chem* 1998; **273**: 33750-33758
25 **Parsons CJ**, Takashima M, Rippe RA. Molecular mechanisms of hepatic fibrogenesis. *J Gastroenterol Hepatol* 2007; **22** Suppl 1: S79-S84
26 **Pinzani M**, Gesualdo L, Sabbah GM, Abboud HE. Effects of platelet-derived growth factor and other polypeptide mitogens on DNA synthesis and growth of cultured rat liver fat-storing cells. *J Clin Invest* 1989; **84**: 1786-1793
27 **Panta GR**, Kaur S, Cavin LG, Cortés ML, Mercurio F, Lothstein L, Sweatman TW, Israel M, Arsura M. ATM and the catalytic subunit of DNA-dependent protein kinase activate NF-kappaB through a common MEK/extracellular signal-regulated kinase/p90(rsk) signaling pathway in response to distinct forms of DNA damage. *Mol Cell Biol* 2004; **24**: 1823-1835
28 **Shah BH**, Farshori MP, Jambusaria A, Catt KJ. Roles of Src and epidermal growth factor receptor transactivation in transient and sustained ERK1/2 responses to gonadotropin-releasing hormone receptor activation. *J Biol Chem* 2003; **278**: 19118-19126
29 **Toledo-Pereyra LH**, Lopez-Neblina F, Reuben JS, Toledo AH, Ward PA. Selectin inhibition modulates Akt/MAPK signaling and chemokine expression after liver ischemia-reperfusion. *J Invest Surg* 2004; **17**: 303-313
30 **Buck M**, Chojkier M. A ribosomal S-6 kinase-mediated signal to C/EBP-beta is critical for the development of liver fibrosis. *PLoS ONE* 2007; **2**: e1372


**S- Editor** Li LF **L- Editor** Ma JY **E- Editor** Zheng XM

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.15.2116

*World J Gastroenterol* 2009 May 7; 15(17): 2116-2124
***World Journal of Gastroenterology*** ISSN 1007-9327


*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.
Supported by A PhD grant from the French Ministry of Foreign Affairs (French Embassy in Beijing) to Ren-Yong Lin, by a project grant from the "Foundation Transplantation" (2005-2006), by a grant from NSFC, No. 30860253 and 30760239, and by the Xinjiang Key-Lab project grants on Echinococcosis, No. XJDX0202-2005-01 and XJDX0202-2007-04
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Received: January 10, 2009 Revised: March 19, 2009
Accepted: March 26, 2009
Published online: May 7, 2009

## 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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Lin RY, Wang JH, Lu XM, Zhou XT, Mantion G, Wen H, Vuitton DA, Richert L. Components of the mitogen-activated protein kinase cascade are activated in hepatic cells by *Echinococcus multilocularis* metacestode. *World J Gastroenterol* 2009; 15(17): 2116-2124 Available from: URL: http://www.wjgnet.com/1007-9327/15/2116.asp DOI: http://dx.doi.org/10.3748/wjg.15.2116

## 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

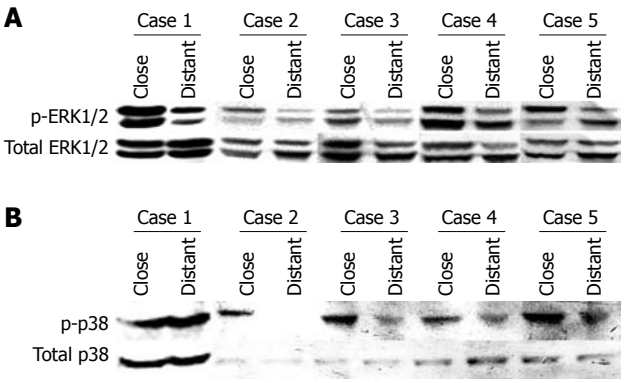


**Figure 1 ERK1/2 (A) and p38 (B) activation in liver samples from five AE patients.** Western immunoblot analyses were performed on lysates with antibodies that recognize phosphorylated and total ERK1/2 respectively (A), and phosphorylated- and total p38, respectively (B). Close: Liver samples close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

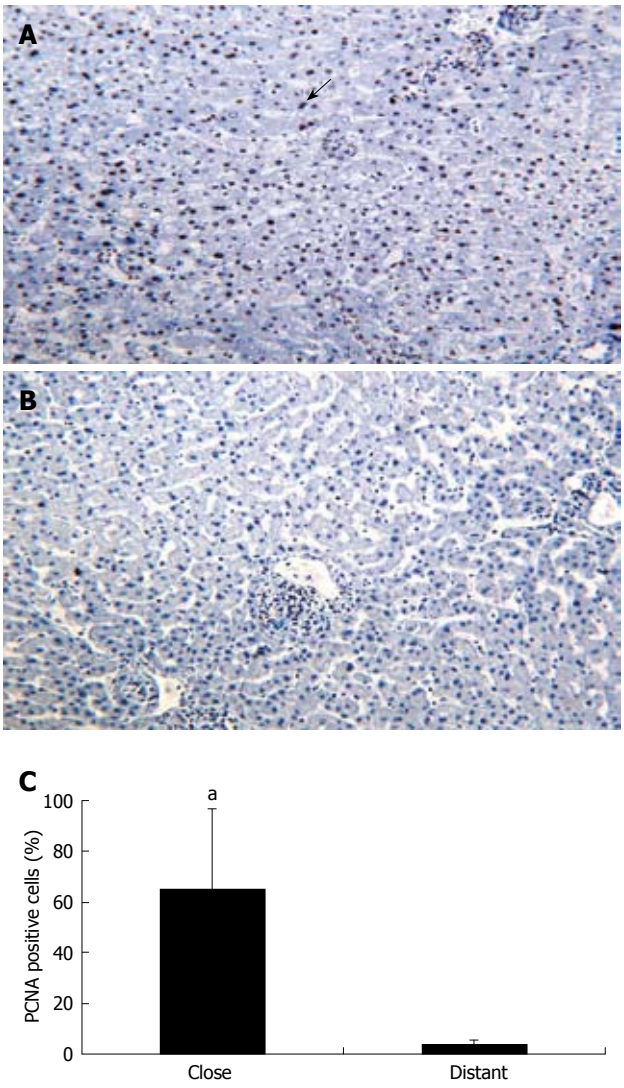


**Figure 2 PCNA expression by hepatic cells in the liver from five patients with AE (immunohistochemical analysis).** A: Hepatic cells close to the parasitic lesions were strongly labeled by the anti-PCNA antibody; all cells with a dark-brown/black nucleus are positive cells; some of them indicated by 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).

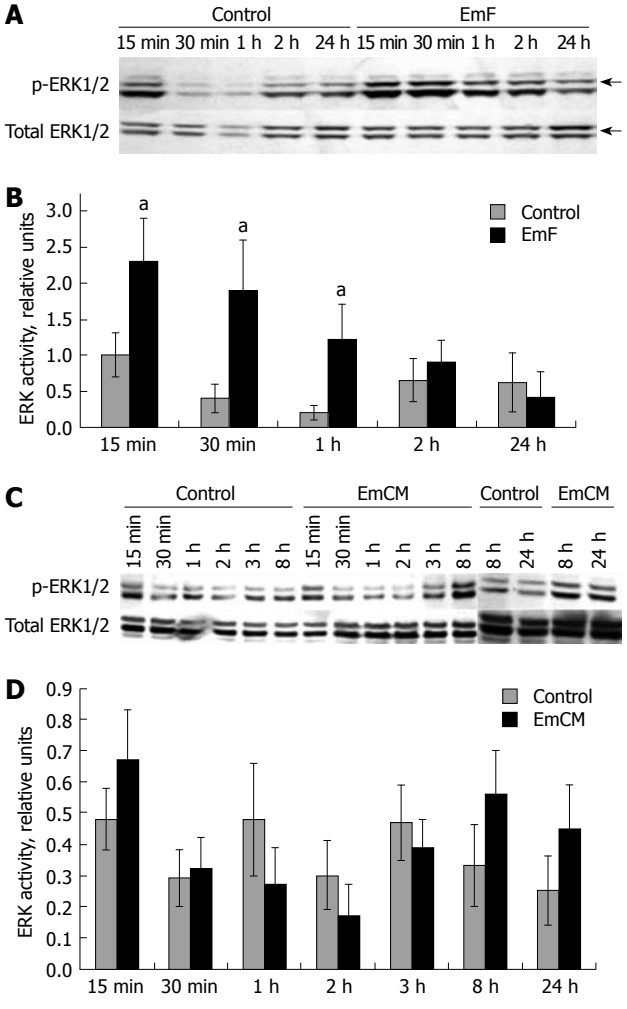


**Figure 3 Time course of EmF- or EmCM- induced phosphorylation of ERK1/2 kinase.** Primary cultures of rat hepatocytes were stimulated with EmF (A, B) or EmCM (C, D) and harvested at the indicated time points (15 min to 24 h). Western immunoblot analyses were performed on lysates with antibodies that 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

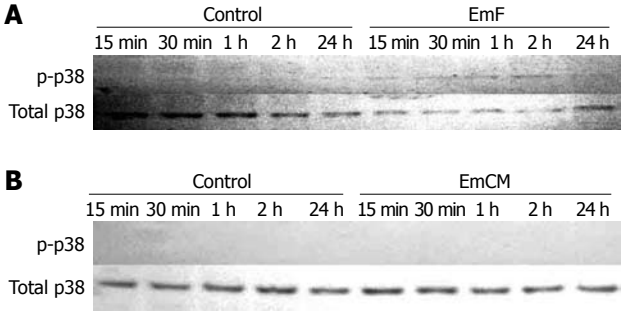


**Figure 4 Time course of EmF- or EmCM-induced phosphorylation of p38 kinase.** Primary cultures of rat hepatocytes were stimulated with EmF (A) or EmCM (B) and harvested at the indicated time points (15 min to 24 h). Western immunoblot analyses were performed on lysates with antibodies that recognize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

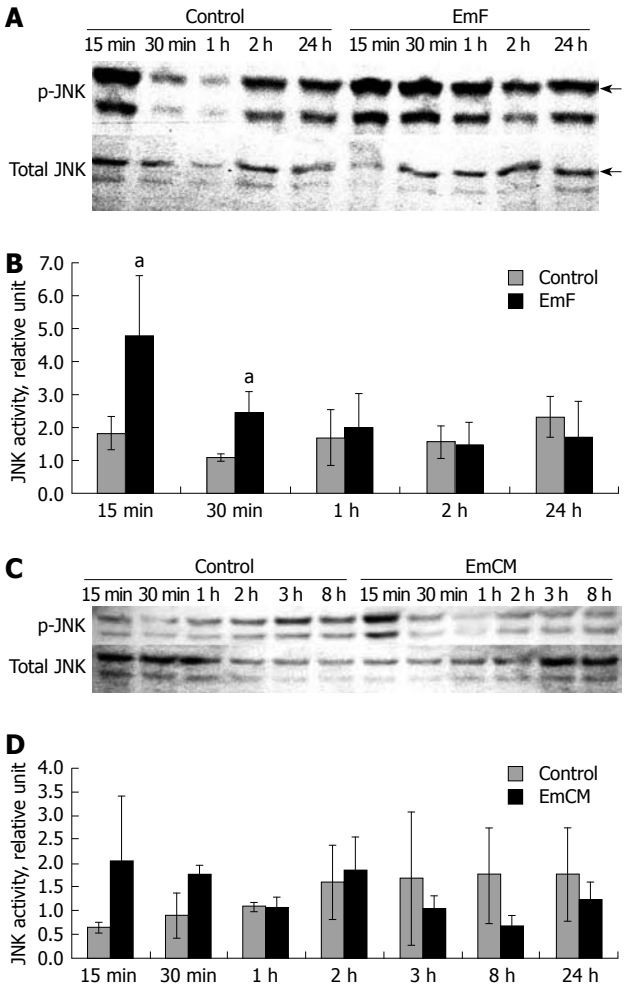


**Figure 5 Time course of EmF- or EmCM-induced phosphorylation of JNK.** Primary cultures of rat hepatocytes were stimulated with EmF (A, B) or EmCM (C, D) and harvested at the indicated time points (15 min to 24 h). Western immunoblot analyses were performed on lysates with antibodies that recognize 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.

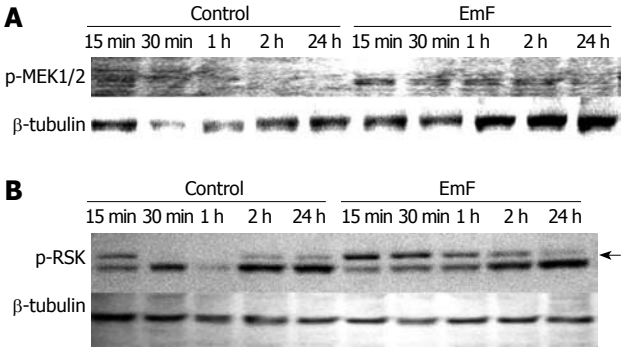


**Figure 6 Time course of EmF-induced phosphorylation of MEK1/2 (A) and RSK (B).** Primary cultures of rat hepatocytes were stimulated with EmF and harvested at the indicated time points (15 min to 24 h). Western immunoblot analyses were performed on lysates with antibodies that recognize phosphorylated (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.

## ACKNOWLEDGMENTS

We would like to thank Professor Klaus Brehm, Institute of Hygiene and Microbiology, University of Würzburg, Germany, for his generous gift of EmCM and his constructive advice and comments on this study, Professor Bernadette Kantelip, head of the Department of Pathology, Besançon University Hospital, for her valuable help in histopathological aspects of the study, and Catherine Guyon and Alexandre Bonet and Hui Liu for their excellent technical assistance.

## 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.

## REFERENCES

1 **Bréchot C**. Pathogenesis of hepatitis B virus-related hepatocellular carcinoma: old and new paradigms. *Gastroenterology* 2004; **127**: S56-S61

2 **Hassan M**, Ghozlan H, Abdel-Kader O. Activation of c-Jun NH2-terminal kinase (JNK) signaling pathway is essential for the stimulation of hepatitis C virus (HCV) non-structural protein 3 (NS3)-mediated cell growth. *Virology* 2005; **333**: 324-336

3 **Schmitz KJ**, Wohlschlaeger J, Lang H, Sotiropoulos GC, Malago M, Steveling K, Reis H, Cicinnati VR, Schmid KW, Baba HA. Activation of the ERK and AKT signalling pathway predicts poor prognosis in hepatocellular carcinoma and ERK activation in cancer tissue is associated with hepatitis C virus infection. *J Hepatol* 2008; **48**: 83-90

4 **Vuitton DA**, Piarroux R, Bresson-Hadni S. Non-viral infectious diseases of the liver. In: Bianchi-Porro G, Cremer M, Krejs G, Ramadori G, Rask-Madsen J, Isselbacher KJ, eds. Hepatology. New York: McGraw-Hill, 1999: 644-667

5 **Craig P**. Echinococcus multilocularis. *Curr Opin Infect Dis* 2003; **16**: 437-444

6 **Bresson-Hadni S**, Delabrousse E, Blagosklonov O, Bartholomot B, Koch S, Miguet JP, André Mantion G, Angèle Vuitton D. Imaging aspects and non-surgical interventional treatment in human alveolar echinococcosis.

*Parasitol Int* 2006; **55** Suppl: S267-S272
7 **Bresson-Hadni S**, Miguet JP, Vuitton DA. Echinococcosis of the liver. In: Bircher J, Benhamou JP, McIntyre N, Rizzetto M, Rodes J, editors. Oxford textbook of clinical hepatology. 2nd ed. Oxford: Oxford University Press, 1999: 1066-1076
8 **Grenard P**, Bresson-Hadni S, El Alaoui S, Chevallier M, Vuitton DA, Ricard-Blum S. Transglutaminase-mediated cross-linking is involved in the stabilization of extracellular matrix in human liver fibrosis. *J Hepatol* 2001; **35**: 367-375
9 **Guerret S**, Vuitton DA, Liance M, Pater C, Carbillet JP. Echinococcus multilocularis: relationship between susceptibility/resistance and liver fibrogenesis in experimental mice. *Parasitol Res* 1998; **84**: 657-667
10 **Ricard-Blum S**, Bresson-Hadni S, Guerret S, Grenard P, Volle PJ, Risteli L, Grimaud JA, Vuitton DA. Mechanism of collagen network stabilization in human irreversible granulomatous liver fibrosis. *Gastroenterology* 1996; **111**: 172-182
11 **Ricard-Blum S**, Bresson-Hadni S, Vuitton DA, Ville G, Grimaud JA. Hydroxypyridinium collagen cross-links in human liver fibrosis: study of alveolar echinococcosis. *Hepatology* 1992; **15**: 599-602
12 **Ricard-Blum S**, Liance M, Houin R, Grimaud JA, Vuitton DA. Covalent cross-linking of liver collagen by pyridinoline increases in the course of experimental alveolar echinococcosis. *Parasite* 1995; **2**: 113-118
13 **Vuitton DA**. The ambiguous role of immunity in echinococcosis: protection of the host or of the parasite? *Acta Trop* 2003; **85**: 119-132
14 **Laurent S**, Stärkel P, Leclercq IA, Lambotte L, Maiter D, Horsmans Y. Molecular events associated with accelerated proliferative response in rat livers when partial hepatectomy is preceded by a sham operation. *Eur J Clin Invest* 2005; **35**: 140-147
15 **Osada S**, Kanematsu M, Imai H, Goshima S, Sugiyama Y. Evaluation of extracellular signal regulated kinase expression and its relation to treatment of hepatocellular carcinoma. *J Am Coll Surg* 2005; **201**: 405-411
16 **Brehm K**, Spiliotis M, Zavala-Góngora R, Konrad C, Frosch M. The molecular mechanisms of larval cestode development: first steps into an unknown world. *Parasitol Int* 2006; **55** Suppl: S15-S21
17 **Hemphill A**, Stettler M, Walker M, Siles-Lucas M, Fink R, Gottstein B. Culture of Echinococcus multilocularis metacestodes: an alternative to animal use. *Trends Parasitol* 2002; **18**: 445-451
18 **Spiliotis M**, Konrad C, Gelmedin V, Tappe D, Brückner S, Mösch HU, Brehm K. Characterisation of EmMPK1, an ERK-like MAP kinase from Echinococcus multilocularis which is activated in response to human epidermal growth factor. *Int J Parasitol* 2006; **36**: 1097-1112
19 **Kepron C**, Novak M, Blackburn BJ. Effect of Echinococcus multilocularis on the origin of acetyl-coA entering the tricarboxylic acid cycle in host liver. *J Helminthol* 2002; **76**: 31-36
20 **Gabrion C**, Walbaum S, al Nahhas S, Mesnil M, Petavy AF. Echinococcus multilocularis protoscoleces and hepatic cell activity in vitro. *Int J Parasitol* 1995; **25**: 127-130
21 **Roux PP**, Blenis J. ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions. *Microbiol Mol Biol Rev* 2004; **68**: 320-344
22 **Schoemaker MH**, Moshage H. Defying death: the hepatocyte's survival kit. *Clin Sci* (Lond) 2004; **107**: 13-25
23 **Ballif BA**, Blenis J. Molecular mechanisms mediating mammalian mitogen-activated protein kinase (MAPK) kinase (MEK)-MAPK cell survival signals. *Cell Growth Differ* 2001; **12**: 397-408
24 **Aroor AR**, Shukla SD. MAP kinase signaling in diverse effects of ethanol. *Life Sci* 2004; **74**: 2339-2364
25 **Gottstein B**, Jacquier P, Bresson-Hadni S, Eckert J. Improved primary immunodiagnosis of alveolar echinococcosis in humans by an enzyme-linked immunosorbent assay using the Em2plus antigen. *J Clin Microbiol* 1993; **31**: 373-376
26 **Hsu MK**, Qiao L, Ho V, Zhang BH, Zhang H, Teoh N, Dent P, Farrell GC. Ethanol reduces p38 kinase activation and cyclin D1 protein expression after partial hepatectomy in rats. *J Hepatol* 2006; **44**: 375-382
27 **Spiliotis M**, Tappe D, Sesterhenn L, Brehm K. Long-term in vitro cultivation of Echinococcus multilocularis metacestodes under axenic conditions. *Parasitol Res* 2004; **92**: 430-432
28 **Richert L**, Binda D, Hamilton G, Viollon-Abadie C, Alexandre E, Bigot-Lasserre D, Bars R, Coassolo P, LeCluyse E. Evaluation of the effect of culture configuration on morphology, survival time, antioxidant status and metabolic capacities of cultured rat hepatocytes. *Toxicol In Vitro* 2002; **16**: 89-99
29 **Carmichael J**, DeGraff WG, Gazdar AF, Minna JD, Mitchell JB. Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing. *Cancer Res* 1987; **47**: 936-942
30 **Rao YP**, Studer EJ, Stravitz RT, Gupta S, Qiao L, Dent P, Hylemon PB. Activation of the Raf-1/MEK/ERK cascade by bile acids occurs via the epidermal growth factor receptor in primary rat hepatocytes. *Hepatology* 2002; **35**: 307-314
31 **Frödin M**, Gammeltoft S. Role and regulation of 90 kDa ribosomal S6 kinase (RSK) in signal transduction. *Mol Cell Endocrinol* 1999; **151**: 65-77
32 **Godeny MD**, Sayeski PP. ERK1/2 regulates ANG II-dependent cell proliferation via cytoplasmic activation of RSK2 and nuclear activation of elk1. *Am J Physiol Cell Physiol* 2006; **291**: C1308-C1317
33 **Fausto N**, Campbell JS, Riehle KJ. Liver regeneration. *Hepatology* 2006; **43**: S45-S53
34 **Taub R**. Liver regeneration: from myth to mechanism. *Nat Rev Mol Cell Biol* 2004; **5**: 836-847
35 **Bresson-Hadni S**, Petitjean O, Monnot-Jacquard B, Heyd B, Kantelip B, Deschaseaux M, Racadot E, Vuitton DA. Cellular localisations of interleukin-1 beta, interleukin-6 and tumor necrosis factor-alpha mRNA in a parasitic granulomatous disease of the liver, alveolar echinococcosis. *Eur Cytokine Netw* 1994; **5**: 461-468
36 **Godot V**, Harraga S, Beurton I, Tiberghien P, Sarciron E, Gottstein B, Vuitton DA. Resistance/susceptibility to Echinococcus multilocularis infection and cytokine profile in humans. II. Influence of the HLA B8, DR3, DQ2 haplotype. *Clin Exp Immunol* 2000; **121**: 491-498
37 **Harraga S**, Godot V, Bresson-Hadni S, Mantion G, Vuitton DA. Profile of cytokine production within the periparasitic granuloma in human alveolar echinococcosis. *Acta Trop* 2003; **85**: 231-236
38 **Zhang S**, Hüe S, Sène D, Penfornis A, Bresson-Hadni S, Kantelip B, Caillat-Zucman S, Vuitton DA. Expression of major histocompatibility complex class I chain-related molecule A, NKG2D, and transforming growth factor-beta in the liver of humans with alveolar echinococcosis: new actors in the tolerance to parasites? *J Infect Dis* 2008; **197**: 1341-1349
39 **Chemale G**, van Rossum AJ, Jefferies JR, Barrett J, Brophy PM, Ferreira HB, Zaha A. Proteomic analysis of the larval stage of the parasite Echinococcus granulosus: causative agent of cystic hydatid disease. *Proteomics* 2003; **3**: 1633-1636
40 **Díaz A**, Ferreira A, Sim RB. Complement evasion by Echinococcus granulosus: sequestration of host factor H in the hydatid cyst wall. *J Immunol* 1997; **158**: 3779-3786
41 **Marco M**, Baz A, Fernandez C, Gonzalez G, Hellman U, Salinas G, Nieto A. A relevant enzyme in granulomatous reaction, active matrix metalloproteinase-9, found in bovine Echinococcus granulosus hydatid cyst wall and fluid. *Parasitol Res* 2006; **100**: 131-139
42 **Gottstein B**, Hemphill A. Echinococcus multilocularis: the

parasite-host interplay. *Exp Parasitol* 2008; **119**: 447-452
43 **Spiliotis M**, Brehm K. Echinococcus multilocularis: identification and molecular characterization of a Ral-like small GTP-binding protein. *Exp Parasitol* 2004; **107**: 163-172
44 **Spiliotis M**, Kroner A, Brehm K. Identification, molecular characterization and expression of the gene encoding the epidermal growth factor receptor orthologue from the fox-tapeworm Echinococcus multilocularis. *Gene* 2003; **323**: 57-65
45 **Zhou HX**, Mo JJ, Chen G, Bao GS, Shi DZ. [Effect of combined pentoxifylline and albendazole against echinococcus multilocularis infection in mice] *Zhongguo Jishengchongxue Yu Jishengchongbing Zazhi* 2006; **24**: 333-336
46 **Zavala-Góngora R**, Derrer B, Gelmedin V, Knaus P, Brehm K. Molecular characterisation of a second structurally unusual AR-Smad without an MH1 domain and a Smad4 orthologue from Echinococcus multilocularis. *Int J Parasitol* 2008; **38**: 161-176
47 **Zavala-Góngora R**, Kroner A, Bernthaler P, Knaus P, Brehm K. A member of the transforming growth factor-beta receptor family from Echinococcus multilocularis is activated by human bone morphogenetic protein 2. *Mol Biochem Parasitol* 2006; **146**: 265-271
48 **Wan YY**, Flavell RA. 'Yin-Yang' functions of transforming growth factor-beta and T regulatory cells in immune regulation. *Immunol Rev* 2007; **220**: 199-213
49 **Dennler S**, Goumans MJ, ten Dijke P. Transforming growth factor beta signal transduction. *J Leukoc Biol* 2002; **71**: 731-740
50 **Moreira RK**. Hepatic stellate cells and liver fibrosis. *Arch Pathol Lab Med* 2007; **131**: 1728-1734

**S- Editor** Tian L **L- Editor** Cant MR **E- Editor** Lin YP

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.15.2125

*World J Gastroenterol* 2009 May 7; 15(17): 2125-2131
***World Journal of Gastroenterology*** ISSN 1007-9327




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

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Supported by The National Natural Science Foundation of China, No. 30760068
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Received: February 7, 2009 Revised: March 12, 2009
Accepted: March 19, 2009
Published online: May 7, 2009

## 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

**Peer reviewer:** Leonard R Johnson, Professor, Department of Physiology, University Tennessee College of Medicine, 894 Union Ave, Memphis, TN 38163, United States

Cai YL, Xu DY, Li XL, Qiu ZX, Jin Z, Xu WX. C-type natriuretic-peptide-potentiated relaxation response of gastric smooth muscle in streptozotocin-induced diabetic rats. *World J Gastroenterol* 2009; 15(17): 2125-2131 Available from: URL: http://www.wjgnet.com/1007-9327/15/2125.asp DOI: http://dx.doi.org/10.3748/wjg.15.2125

## 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-

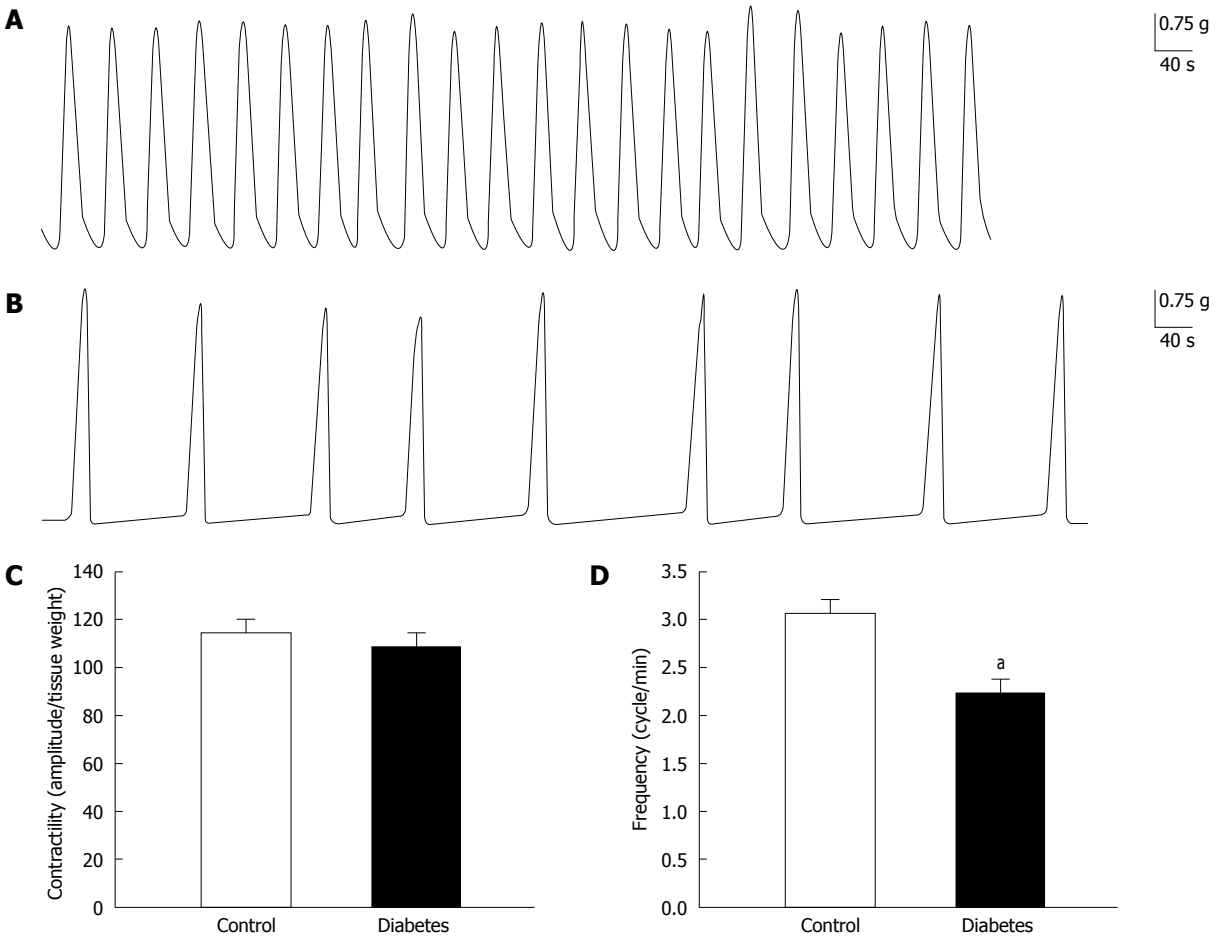


**Figure 1 Comparison of gastric smooth muscle contractilities between normal and diabetic rats.** A, B: The row traces gastric smooth muscle spontaneous contractions in normal and diabetic rats; C, D: Summary of the contractility in normal and diabetic rats. The contractility per weight of gastric smooth 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

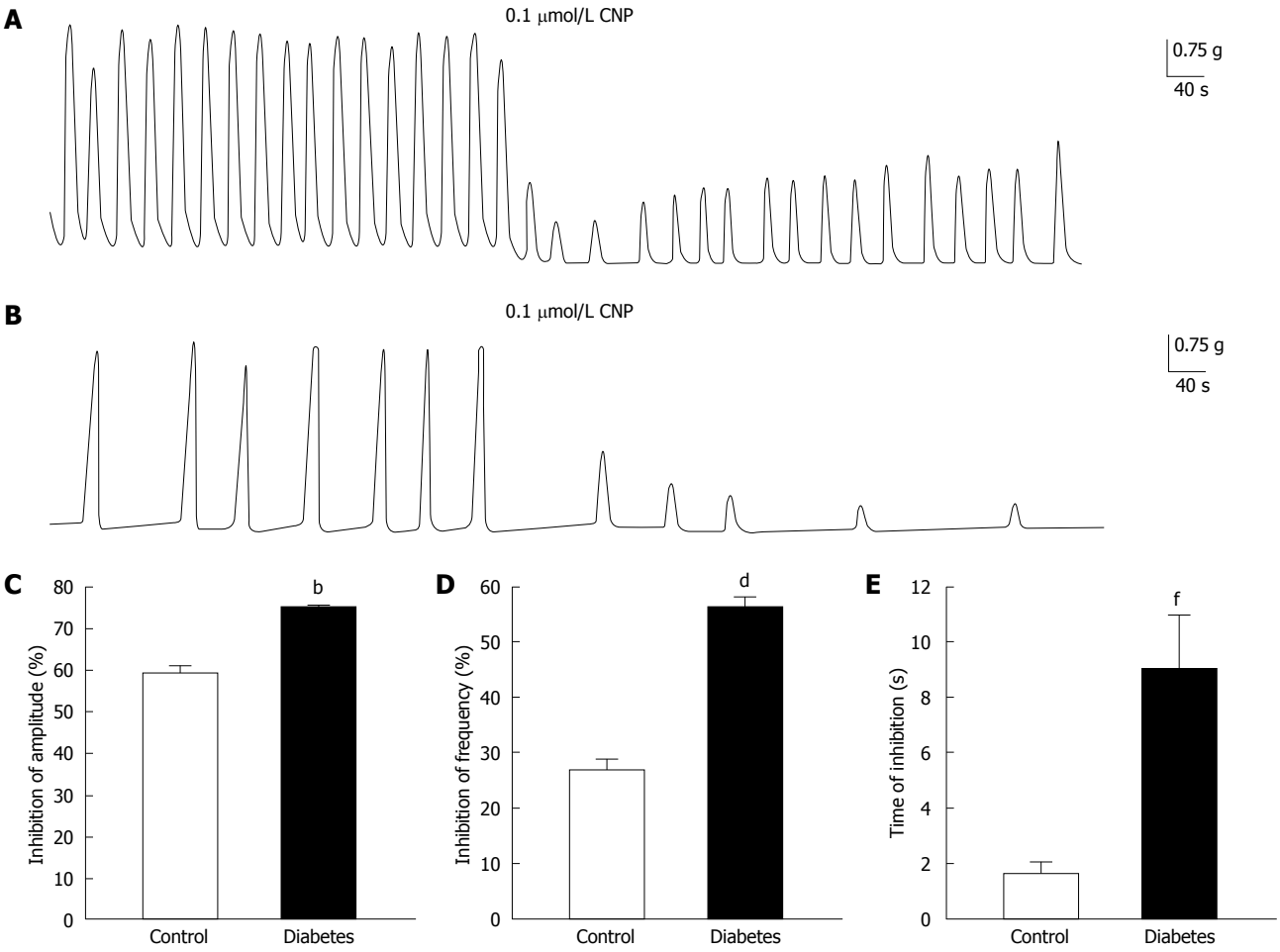


**Figure 2 The sensitivity of gastric smooth muscle to CNP.** A, B: The row traces gastric smooth muscle spontaneous contractions in response to CNP in normal and diabetic rats; C-E: Summary of the contractility in response to CNP in normal and diabetic rats. CNP induced relaxation of gastric antral 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),

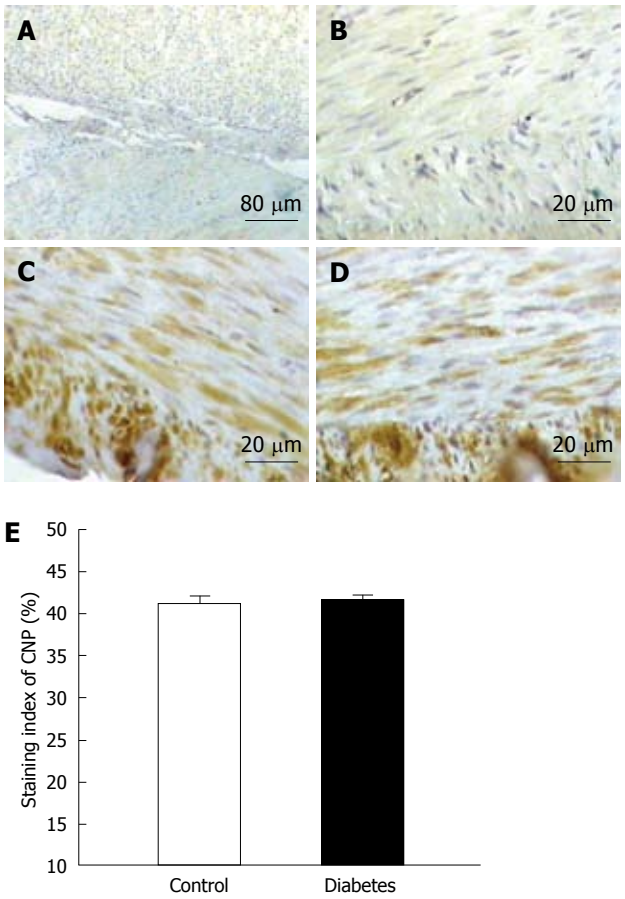


**Figure 3 CNP expression in gastric tissues in normal and diabetic rats.** A-D: CNP expression in gastric smooth muscle in normal and diabetic rats. In negative controls CNP was not expressed in normal and diabetic rats (A, B) and the CNP immunopositive brown granules were mainly expressed in gastric 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).

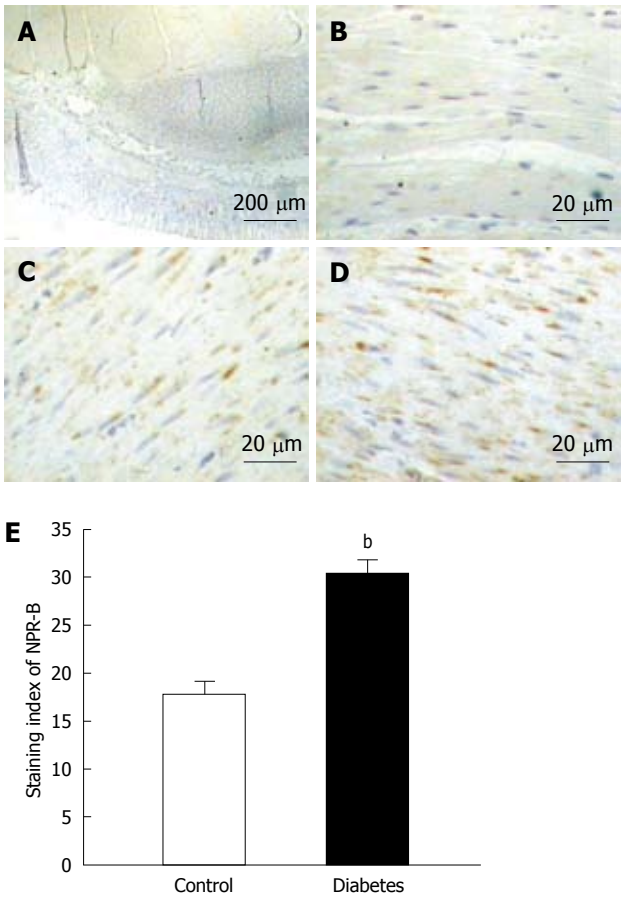


**Figure 4 NPR-B expression in gastric tissues in normal and diabetic rats.** A-D: NPR-B expression in gastric smooth muscle in normal and diabetic rats. There was no NPR-B immunopositive expression in negative controls of normal and diabetic rats (A, B). The NPR-B immunopositive brown granules were 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.

## REFERENCES


1 **Parkman HP**, Hasler WL, Fisher RS. American Gastroenterological Association technical review on the diagnosis and treatment of gastroparesis. *Gastroenterology* 2004; **127**: 1592-1622

2 **Camilleri M**. Advances in diabetic gastroparesis. *Rev Gastroenterol Disord* 2002; **2**: 47-56

3 **Qi HB**, Luo JY, Dai XG, Wang XQ. A study on motility in patients with diabetic gastroparesis. *Xin Xiaohuabingxue Zazhi* 1997; **5**: 661-662

4 **Quigley EM**. The evaluation of gastrointestinal function in diabetic patients. *World J Gastroenterol* 1999; **5**: 277-282

5 **Bult H**, Boeckxstaens GE, Pelckmans PA, Jordaens FH, Van Maercke YM, Herman AG. Nitric oxide as an inhibitory non-adrenergic non-cholinergic neurotransmitter. *Nature* 1990; **345**: 346-347

6 **Endo K**, Matsumoto T, Kobayashi T, Kasuya Y, Kamata K. Diabetes-related changes in contractile responses of stomach fundus to endothelin-1 in streptozotocin-induced diabetic rats. *J Smooth Muscle Res* 2005; **41**: 35-47

7 **de Bold AJ**, Borenstein HB, Veress AT, Sonnenberg H. A rapid and potent natriuretic response to intravenous injection of atrial myocardial extract in rats. *Life Sci* 1981; **28**: 89-94

8 **Maack T**, Suzuki M, Almeida FA, Nussenzveig D, Scarborough RM, McEnroe GA, Lewicki JA. Physiological role of silent receptors of atrial natriuretic factor. *Science* 1987; **238**: 675-678

9 **Schulz S**, Singh S, Bellet RA, Singh G, Tubb DJ, Chin H, Garbers DL. The primary structure of a plasma membrane guanylate cyclase demonstrates diversity within this new receptor family. *Cell* 1989; **58**: 1155-1162

10 **Chang MS**, Lowe DG, Lewis M, Hellmiss R, Chen E, Goeddel DV. Differential activation by atrial and brain natriuretic peptides of two different receptor guanylate cyclases. *Nature* 1989; **341**: 68-72

11 **Chinkers M**, Garbers DL, Chang MS, Lowe DG, Chin HM, Goeddel DV, Schulz S. A membrane form of guanylate cyclase is an atrial natriuretic peptide receptor. *Nature* 1989; **338**: 78-83

12 **Koller KJ**, Lowe DG, Bennett GL, Minamino N, Kangawa K, Matsuo H, Goeddel DV. Selective activation of the B natriuretic peptide receptor by C-type natriuretic peptide (CNP). *Science* 1991; **252**: 120-123

13 **Koller KJ**, Goeddel DV. Molecular biology of the natriuretic peptides and their receptors. *Circulation* 1992; **86**: 1081-1088

14 **Li CH**, Yang ZW, Yin ZR, Jin Z, Xing DG, Piao LH, Kim YC, Xu WX. Relationship between atrial natriuretic peptide-immunoreactive cells and microvessels in rat gastric mucosa. *Acta Pharmacol Sin* 2006; **27**: 205-211

15 **Gower WR Jr**, Salhab KF, Foulis WL, Pillai N, Bundy JR, Vesely DL, Fabri PJ, Dietz JR. Regulation of atrial natriuretic peptide gene expression in gastric antrum by fasting. *Am J Physiol Regul Integr Comp Physiol* 2000; **278**: R770-R780

16 **Gower WR Jr**, McCuen RW, Arimura A, Coy DA, Dietz JR, Landon CS, Schubert ML. Reciprocal paracrine pathways

link atrial natriuretic peptide and somatostatin secretion in the antrum of the stomach. *Regul Pept* 2003; **110**: 101-106
17 **Guo HS**, Jin Z, Jin ZY, Li ZH, Cui YF, Wang ZY, Xu WX. Comparative study in the effect of C-type natriuretic peptide on gastric motility in various animals. *World J Gastroenterol* 2003; **9**: 547-552
18 **Guo HS**, Cui X, Cui YG, Kim SZ, Cho KW, Li ZL, Xu WX. Inhibitory effect of C-type natriuretic peptide on spontaneous contraction in gastric antral circular smooth muscle of rat. *Acta Pharmacol Sin* 2003; **24**: 1021-1026
19 **Guo HS**, Cai ZX, Zheng HF, Li XL, Cui YF, Wang ZY, Xu WX, Lee SJ, Kim YC. Role of calcium-activated potassium currents in CNP-induced relaxation of gastric antral circular smooth muscle in guinea pigs. *World J Gastroenterol* 2003; **9**: 2054-2059
20 **Potter LR**, Hunter T. Guanylyl cyclase-linked natriuretic peptide receptors: structure and regulation. *J Biol Chem* 2001; **276**: 6057-6060
21 **Stepan H**, Leitner E, Bader M, Walther T. Organ-specific mRNA distribution of C-type natriuretic peptide in neonatal and adult mice. *Regul Pept* 2000; **95**: 81-85
22 **Rambotti MG**, Giambanco I, Spreca A. Detection of guanylate cyclases A and B stimulated by natriuretic peptides in gastrointestinal tract of rat. *Histochem J* 1997; **29**: 117-126
23 **Christoffersen C**, Bartels ED, Nielsen LB. Heart specific up-regulation of genes for B-type and C-type natriuretic peptide receptors in diabetic mice. *Eur J Clin Invest* 2006; **36**: 69-75
24 **Shin SJ**, Wen JD, Lee YJ, Chen IH, Tsai JH. Increased C-type natriuretic peptide mRNA expression in the kidney of diabetic rats. *J Endocrinol* 1998; **158**: 35-42
25 **Kim SZ**, Kim SH, Park JK, Koh GY, Cho KW. Presence and biological activity of C-type natriuretic peptide-dependent guanylate cyclase-coupled receptor in the penile corpus cavernosum. *J Urol* 1998; **159**: 1741-1746
26 **Sudoh T**, Minamino N, Kangawa K, Matsuo H. C-type natriuretic peptide (CNP): a new member of natriuretic peptide family identified in porcine brain. *Biochem Biophys Res Commun* 1990; **168**: 863-870
27 **Itaba S**, Chijiiwa Y, Matsuzaka H, Motomura Y, Nawata H. Presence of C-type natriuretic peptide (CNP) in guinea pig caecum: role and mechanisms of CNP in circular smooth muscle relaxation. *Neurogastroenterol Motil* 2004; **16**: 375-382
28 **Kim JH**, Jeon GJ, Kim SZ, Cho KW, Kim SH. C-type natriuretic peptide system in rabbit colon. *Peptides* 2001; **22**: 2061-2068
29 **Xue L**, Suzuki H. Electrical responses of gastric smooth muscles in streptozotocin-induced diabetic rats. *Am J Physiol* 1997; **272**: G77-G83
30 **Takano H**, Imaeda K, Koshita M, Xue L, Nakamura H, Kawase Y, Hori S, Ishigami T, Kurono Y, Suzuki H. Alteration of the properties of gastric smooth muscle in the genetically hyperglycemic OLETF rat. *J Auton Nerv Syst* 1998; **70**: 180-188
31 **Ordög T**, Takayama I, Cheung WK, Ward SM, Sanders KM. Remodeling of networks of interstitial cells of Cajal in a murine model of diabetic gastroparesis. *Diabetes* 2000; **49**: 1731-1739
32 **Carvajal JA**, Germain AM, Huidobro-Toro JP, Weiner CP. Molecular mechanism of cGMP-mediated smooth muscle relaxation. *J Cell Physiol* 2000; **184**: 409-420

**S- Editor** Li LF **L- Editor** Logan S **E- Editor** Zheng XM

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.15.2132

*World J Gastroenterol* 2009 May 7; 15(17): 2132-2138
***World Journal of Gastroenterology*** ISSN 1007-9327


*BRIEF ARTICLES*

# Hyperferritinemia is a risk factor for steatosis in chronic liver disease

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Received: July 6, 2008 Revised: April 3, 2009
Accepted: April 10, 2009
Published online: May 7, 2009

## 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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Licata A, Nebbia ME, Cabibbo G, Lo Iacono G, Barbaria F, Brucato V, Alessi N, Porrovecchio S, Di Marco V, Craxì A, Cammà C. Hyperferritinemia is a risk factor for steatosis in chronic liver disease. *World J Gastroenterol* 2009; 15(17): 2132-2138 Available from: URL: http://www.wjgnet.com/1007-9327/15/2132.asp DOI: http://dx.doi.org/10.3748/wjg.15.2132

## 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 | - | - |

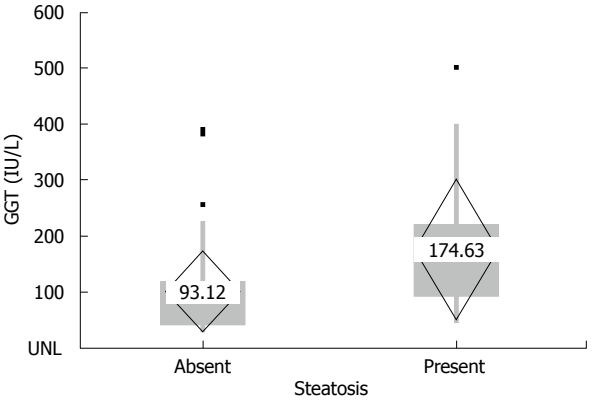


**Figure 1 Baseline GGT levels according to steatosis in 124 non-obese, non-alcoholic patients without hereditary haemochromatosis (HH).**

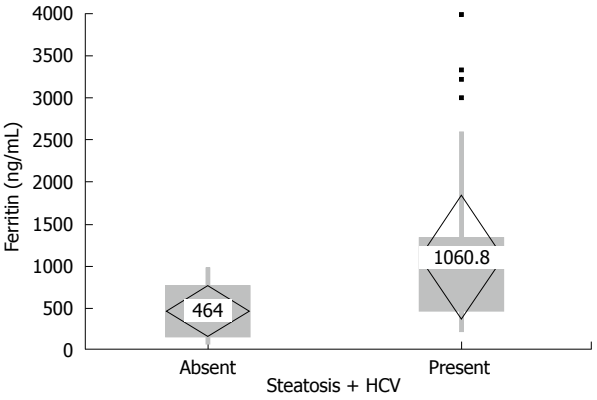


**Figure 2 Baseline ferritin levels according to steatosis and HCV infection status in 124 non-obese, non-alcoholic patients without hereditary haemochromatosis (HH).**

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.

## ACKNOWLEDGMENTS

The authors thank Warren Blumberg for his forbearance in editing the manuscript.

## 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.

## REFERENCES


1 **Le Page L**, Leflon P, Mahévas M, Duhaut P, Smail A, Salle V, Cevallos R, Ducroix JP. [Aetiological spectrum of hyperferritinemia] *Rev Med Interne* 2005; **26**: 368-373

2 **Lee MH**, Means RT Jr. Extremely elevated serum ferritin levels in a university hospital: associated diseases and clinical significance. *Am J Med* 1995; **98**: 566-571

3 **Feder JN**, Gnirke A, Thomas W, Tsuchihashi Z, Ruddy DA, Basava A, Dormishian F, Domingo R Jr, Ellis MC, Fullan A, Hinton LM, Jones NL, Kimmel BE, Kronmal GS, Lauer P, Lee VK, Loeb DB, Mapa FA, McClelland E, Meyer NC, Mintier GA, Moeller N, Moore T, Morikang E, Prass CE, Quintana L, Starnes SM, Schatzman RC, Brunke KJ, Drayna DT, Risch NJ, Bacon BR, Wolff RK. A novel MHC class I-like gene is mutated in patients with hereditary haemochromatosis. *Nat Genet* 1996; **13**: 399-408

4 **Beutler E**, Gelbart T, West C, Lee P, Adams M, Blackstone R, Pockros P, Kosty M, Venditti CP, Phatak PD, Seese NK, Chorney KA, Ten Elshof AE, Gerhard GS, Chorney M. Mutation analysis in hereditary hemochromatosis. *Blood Cells Mol Dis* 1996; **22**: 187-194; discussion 194a-194b

5 **Candore G**, Mantovani V, Balistreri CR, Lio D, Colonna-Romano G, Cerreta V, Carru C, Deiana L, Pes G, Menardi G, Perotti L, Miotti V, Bevilacqua E, Amoroso A, Caruso C. Frequency of the HFE gene mutations in five Italian populations. *Blood Cells Mol Dis* 2002; **29**: 267-273

6 **Marchesini G**, Brizi M, Bianchi G, Tomassetti S, Bugianesi E, Lenzi M, McCullough AJ, Natale S, Forlani G, Melchionda N. Nonalcoholic fatty liver disease: a feature of the metabolic syndrome. *Diabetes* 2001; **50**: 1844-1850

7 **Bonkovsky HL**, Banner BF, Rothman AL. Iron and chronic viral hepatitis. *Hepatology* 1997; **25**: 759-768

8 **Sebastiani G**, Vario A, Ferrari A, Pistis R, Noventa F, Alberti A. Hepatic iron, liver steatosis and viral genotypes in patients with chronic hepatitis C. *J Viral Hepat* 2006; **13**: 199-205

9 **Van Thiel DH**, Friedlander L, Fagiuoli S, Wright HI, Irish W, Gavaler JS. Response to interferon alpha therapy is influenced by the iron content of the liver. *J Hepatol* 1994; **20**: 410-415

10 **Olynyk JK**, Reddy KR, Di Bisceglie AM, Jeffers LJ, Parker TI, Radick JL, Schiff ER, Bacon BR. Hepatic iron concentration as a predictor of response to interferon alfa therapy in chronic hepatitis C. *Gastroenterology* 1995; **108**: 1104-1109

11 **D'Souza RF**, Feakins R, Mears L, Sabin CA, Foster GR. Relationship between serum ferritin, hepatic iron staining, diabetes mellitus and fibrosis progression in patients with chronic hepatitis C. *Aliment Pharmacol Ther* 2005; **21**: 519-524

12 **Lonardo A**, Adinolfi LE, Loria P, Carulli N, Ruggiero G, Day CP. Steatosis and hepatitis C virus: mechanisms and significance for hepatic and extrahepatic disease. *Gastroenterology* 2004; **126**: 586-597

13 **Adinolfi LE**, Gambardella M, Andreana A, Tripodi MF, Utili R, Ruggiero G. Steatosis accelerates the progression of liver damage of chronic hepatitis C patients and correlates with specific HCV genotype and visceral obesity. *Hepatology* 2001; **33**: 1358-1364

14 **Cammà C**, Bruno S, Di Marco V, Di Bona D, Rumi M, Vinci M, Rebucci C, Cividini A, Pizzolanti G, Minola E, Mondelli MU, Colombo M, Pinzello G, Craxì A. Insulin resistance is associated with steatosis in nondiabetic patients with genotype 1 chronic hepatitis C. *Hepatology* 2006; **43**: 64-71

15 **Hwang SJ**, Luo JC, Chu CW, Lai CR, Lu CL, Tsay SH, Wu JC, Chang FY, Lee SD. Hepatic steatosis in chronic hepatitis C virus infection: prevalence and clinical correlation. *J Gastroenterol Hepatol* 2001; **16**: 190-195

16 **Ford ES**, Cogswell ME. Diabetes and serum ferritin concentration among U.S. adults. *Diabetes Care* 1999; **22**: 1978-1983

17 **Paris R**. Association of hepatitis C and diabetes mellitus. *Ann Intern Med* 2001; **135**: 141-142

18 **James OF**, Day CP. Non-alcoholic steatohepatitis (NASH): a disease of emerging identity and importance. *J Hepatol* 1998; **29**: 495-501

19 **Younossi ZM**, Gramlich T, Bacon BR, Matteoni CA, Boparai N, O'Neill R, McCullough AJ. Hepatic iron and nonalcoholic fatty liver disease. *Hepatology* 1999; **30**: 847-850

20 **Angulo P**, Keach JC, Batts KP, Lindor KD. Independent predictors of liver fibrosis in patients with nonalcoholic steatohepatitis. *Hepatology* 1999; **30**: 1356-1362

21 **George DK**, Goldwurm S, MacDonald GA, Cowley LL, Walker NI, Ward PJ, Jazwinska EC, Powell LW. Increased hepatic iron concentration in nonalcoholic steatohepatitis is associated with increased fibrosis. *Gastroenterology* 1998; **114**: 311-318

22 **Bonkovsky HL**, Jawaid Q, Tortorelli K, LeClair P, Cobb J, Lambrecht RW, Banner BF. Non-alcoholic steatohepatitis and iron: increased prevalence of mutations of the HFE gene in non-alcoholic steatohepatitis. *J Hepatol* 1999; **31**: 421-429

23 **Mendler MH**, Turlin B, Moirand R, Jouanolle AM, Sapey T, Guyader D, Le Gall JY, Brissot P, David V, Deugnier Y. Insulin resistance-associated hepatic iron overload. *Gastroenterology* 1999; **117**: 1155-1163

24 **Tavill AS**. Diagnosis and management of hemochromatosis. *Hepatology* 2001; **33**: 1321-1328

25 **Siegelman ES**, Rosen MA. Imaging of hepatic steatosis. *Semin Liver Dis* 2001; **21**: 71-80

26 **Ishak KG**. Chronic hepatitis: morphology and nomenclature. *Mod Pathol* 1994; **7**: 690-713

27 SAS Technical Report, SAS/STAS software: Changes & enhancement, release 6.07. North Carolina: SAS Institute Inc, 1992

28 **Campo S**, Restuccia T, Villari D, Raffa G, Cucinotta D, Squadrito G, Pollicino T, Raimondo G. Analysis of haemochromatosis gene mutations in a population from the Mediterranean Basin. *Liver* 2001; **21**: 233-236

29 **Arber N**, Konikoff FM, Moshkowitz M, Baratz M, Hallak A, Santo M, Halpern Z, Weiss H, Gilat T. Increased serum iron and iron saturation without liver iron accumulation distinguish chronic hepatitis C from other chronic liver diseases. *Dig Dis Sci* 1994; **39**: 2656-2659

30 **Riggio O**, Montagnese F, Fiore P, Folino S, Giambartolomei S, Gandin C, Merli M, Quinti I, Violante N, Caroli S, Senofonte O, Capocaccia L. Iron overload in patients with chronic viral hepatitis: how common is it? *Am J Gastroenterol* 1997; **92**: 1298-1301

31 **Olynyk JK**, Clarke SL. Iron overload impairs pro-inflammatory cytokine responses by Kupffer cells. *J Gastroenterol Hepatol* 2001; **16**: 438-444

32 **Weiss G**. Iron and immunity: a double-edged sword. *Eur J Clin Invest* 2002; **32** Suppl 1: 70-78

33 **Rigamonti C**, Andorno S, Maduli E, Morelli S, Pittau S, Nicosia G, Boldorini R, Sartori M. Iron, hepatic stellate cells and fibrosis in chronic hepatitis C. *Eur J Clin Invest* 2002; **32** Suppl 1: 28-35

34 **Martinelli AL**, Ramalho LN, Zucoloto S. Hepatic stellate cells in hepatitis C patients: relationship with liver iron deposits and severity of liver disease. *J Gastroenterol Hepatol* 2004; **19**: 91-98

35 **Fargion S**, Mattioli M, Fracanzani AL, Sampietro M, Tavazzi D, Fociani P, Taioli E, Valenti L, Fiorelli G. Hyperferritinemia, iron overload, and multiple metabolic alterations identify patients at risk for nonalcoholic steatohepatitis. *Am J Gastroenterol* 2001; **96**: 2448-2455

36 **Bugianesi E**, Manzini P, D'Antico S, Vanni E, Longo F, Leone N, Massarenti P, Piga A, Marchesini G, Rizzetto M. Relative contribution of iron burden, HFE mutations, and insulin resistance to fibrosis in nonalcoholic fatty liver. *Hepatology* 2004; **39**: 179-187

37 **Lonardo A**, Loria P, Adinolfi LE, Carulli N, Ruggiero G. Hepatitis C and steatosis: a reappraisal. *J Viral Hepat* 2006; **13**: 73-80

38 **Rubbia-Brandt L**, Fabris P, Paganin S, Leandro G, Male PJ, Giostra E, Carlotto A, Bozzola L, Smedile A, Negro F. Steatosis affects chronic hepatitis C progression in a genotype specific way. *Gut* 2004; **53**: 406-412

39 **Malaguarnera L**, Madeddu R, Palio E, Arena N, Malaguarnera M. Heme oxygenase-1 levels and oxidative stress-related parameters in non-alcoholic fatty liver disease patients. *J Hepatol* 2005; **42**: 585-591

40 **Zelber-Sagi S**, Nitzan-Kaluski D, Halpern Z, Oren R. NAFLD and hyperinsulinemia are major determinants of serum ferritin levels. *J Hepatol* 2007; **46**: 700-707

41 **Trombini P**, Piperno A. Ferritin, metabolic syndrome and NAFLD: elective attractions and dangerous liaisons. *J Hepatol* 2007; **46**: 549-552

42 **Fernández-Real JM**, Ricart-Engel W, Arroyo E, Balançá R, Casamitjana-Abella R, Cabrero D, Fernández-Castañer M, Soler J. Serum ferritin as a component of the insulin resistance syndrome. *Diabetes Care* 1998; **21**: 62-68

43 **Tuomainen TP**, Nyyssönen K, Salonen R, Tervahauta A, Korpela H, Lakka T, Kaplan GA, Salonen JT. Body iron stores are associated with serum insulin and blood glucose concentrations. Population study in 1,013 eastern Finnish men. *Diabetes Care* 1997; **20**: 426-428

44 **Jiang R**, Manson JE, Meigs JB, Ma J, Rifai N, Hu FB. Body iron stores in relation to risk of type 2 diabetes in apparently healthy women. *JAMA* 2004; **291**: 711-717

45 **Lecube A**, Hernández C, Genescà J, Esteban JI, Jardí R, García L, Simó R. Diabetes is the main factor accounting for the high ferritin levels detected in chronic hepatitis C virus infection. *Diabetes Care* 2004; **27**: 2669-2675

46 **Pagliaro L**, D'Amico G, Luca A, Pasta L, Politi F, Aragona E, Malizia G. Portal hypertension: diagnosis and treatment. *J Hepatol* 1995; **23** Suppl 1: 36-44

47 **Stranges S**, Dorn JM, Muti P, Freudenheim JL, Farinaro E, Russell M, Nochajski TH, Trevisan M. Body fat distribution, relative weight, and liver enzyme levels: a population-based study. *Hepatology* 2004; **39**: 754-763

48 **Hourigan LF**, Macdonald GA, Purdie D, Whitehall VH, Shorthouse C, Clouston A, Powell EE. Fibrosis in chronic hepatitis C correlates significantly with body mass index and steatosis. *Hepatology* 1999; **29**: 1215-1219


**S- Editor** Tian L **L- Editor** Kerr C **E- Editor** Zheng XM

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.15.2139

*World J Gastroenterol* 2009 May 7; 15(17): 2139-2144
***World Journal of Gastroenterology*** ISSN 1007-9327




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

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Received: February 6, 2009 Revised: March 8, 2009
Accepted: March 15, 2009
Published online: May 7, 2009

## 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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Liang XM, Tang GY, Cheng YS, Zhou B. Evaluation of a rabbit rectal VX2 carcinoma model using computed tomography and magnetic resonance imaging. *World J Gastroenterol* 2009; 15(17): 2139-2144 Available from: URL: http://www.wjgnet.com/1007-9327/15/2139.asp DOI: http://dx.doi.org/10.3748/wjg.15.2139

## 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 $T_1$W-TSE, gadolinium-enhanced $T_1$W-TSE, and $T_2$W-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.

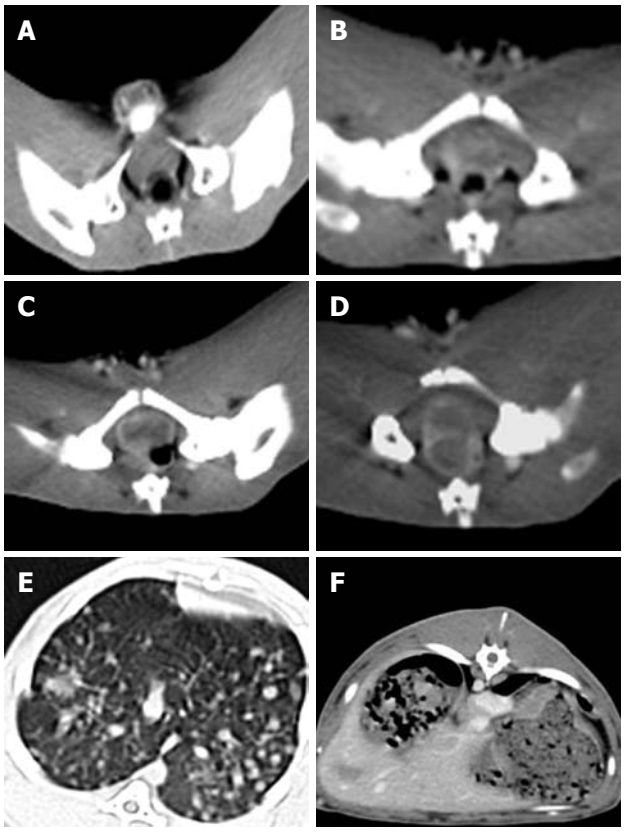


**Figure 1 CT enhancement scanning images of rectal wall 2(A), 3(B), 4(C), and 5(D) wk after VX2 cell implantation in the experimental rabbits, and images of metastatic nodes detected in the lung (E) and liver (F), respectively.**

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.

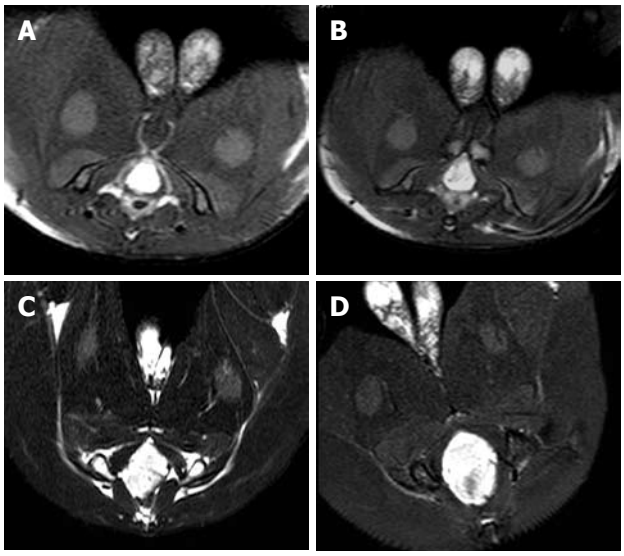


**Figure 2 MRI of T2-TSE-SPAIR 2(A), 3(B), 4(C), and 5(D) wk after VX2 cell implantation in the experimental rabbits.**

### *MRI detection*

MRI showed that the signal of VX2 tumor was low on $T_1$-weighted imaging ($T_1$WI), resulting in the detection of an indistinct boundary, and high on $T_2$-weighted imaging ($T_2$WI), allowing clear visualization of the boundary. In addition, the signal of VX2 tumor in PD sequence was higher than that on $T_1$WI. 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

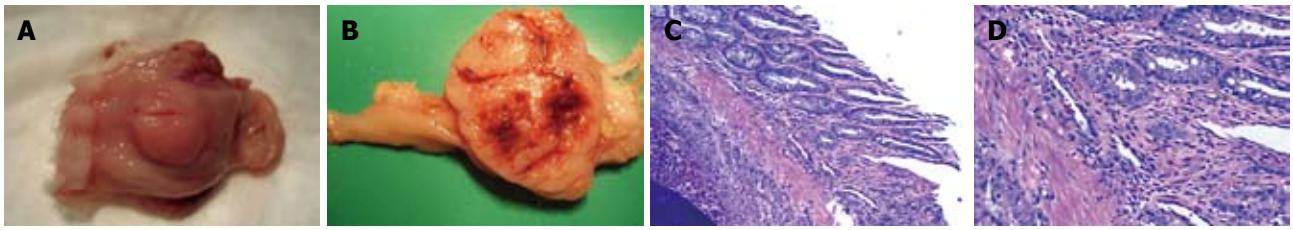


**Figure 3 Characteristics of isolated rectal carcinoma specimens (A, B) and light microscope images of HE stained tissue sections (C, × 40; D, × 100).**

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. $T_1$WI can be used to evaluate fatty infiltration around the rectum, while $T_2$WI 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.

## REFERENCES


1 **Low G**, Tho LM, Leen E, Wiebe E, Kakumanu S, McDonald AC, Poon FW. The role of imaging in the pre-operative staging and post-operative follow-up of rectal cancer. *Surgeon* 2008; **6**: 222-231

2 **Adeyemo D**, Hutchinson R. Preoperative staging of rectal cancer: pelvic MRI plus abdomen and pelvic CT. Does extrahepatic abdomen imaging matter? A case for routine thoracic CT. *Colorectal Dis* 2009; **11**: 259-263

3 **Iafrate F**, Laghi A, Paolantonio P, Rengo M, Mercantini P, Ferri M, Ziparo V, Passariello R. Preoperative staging of rectal cancer with MR Imaging: correlation with surgical and histopathologic findings. *Radiographics* 2006; **26**: 701-714

4 **Hinoi T**, Akyol A, Theisen BK, Ferguson DO, Greenson JK, Williams BO, Cho KR, Fearon ER. Mouse model of colonic adenoma-carcinoma progression based on somatic Apc inactivation. *Cancer Res* 2007; **67**: 9721-9730

5 **Colnot S**, Niwa-Kawakita M, Hamard G, Godard C, Le Plenier S, Houbron C, Romagnolo B, Berrebi D, Giovannini M, Perret C. Colorectal cancers in a new mouse model of familial adenomatous polyposis: influence of genetic and environmental modifiers. *Lab Invest* 2004; **84**: 1619-1630

6 **Mori F**, Piro FR, Della Rocca C, Mesiti G, Giampaoli S, Silvestre G, Lazzaro D. Survivin and Cyclooxygenase-2 are co-expressed in human and mouse colon carcinoma and in terminally differentiated colonocytes. *Histol Histopathol* 2007; **22**: 61-77

7 **Virmani S**, Harris KR, Szolc-Kowalska B, Paunesku T, Woloschak GE, Lee FT, Lewandowski RJ, Sato KT, Ryu RK, Salem R, Larson AC, Omary RA. Comparison of two different methods for inoculating VX2 tumors in rabbit livers and hind limbs. *J Vasc Interv Radiol* 2008; **19**: 931-936

8 **Choi JA**, Kang EY, Kim HK, Song IC, Kim YI, Kang HS. Evolution of VX2 carcinoma in rabbit tibia: magnetic resonance imaging with pathologic correlation. *Clin Imaging* 2008; **32**: 128-135

9 **Chen J**, Yao Q, Li D, Zhang B, Li L, Wang L. Chemotherapy targeting regional lymphatic tissues to treat rabbits bearing VX2 tumor in the mammary glands. *Cancer Biol Ther* 2008; **7**: 721-725

10 **Hu HY**, Li Q, Han ZG, Kang DQ. [Efficacy and safety of percutaneous microwave coagulation therapy for experimental vx2 lung cancer in rabbits] *Aizheng* 2007; **26**: 942-946

11 **Kreuter KA**, El-Abbadi N, Shbeeb A, Tseng L, Mahon SB, Narula N, Burney T, Colt H, Brenner M. Development of a rabbit pleural cancer model by using VX2 tumors. *Comp Med* 2008; **58**: 287-293

12 **Lee JM**, Kim SW, Chung GH, Lee SY, Han YM, Kim CS. Open radio-frequency thermal ablation of renal VX2 tumors in a rabbit model using a cooled-tip electrode: feasibility, safety, and effectiveness. *Eur Radiol* 2003; **13**: 1324-1332

13 **Wang XD**, Zhang JX, Shang D, Zhen QC. Establishment and biological characterization of rectal cancer by transplant VX2 in a rabbit. *Zhong Liu* 2006; **26**: 788-789

14 **Yano H**, Saito Y, Takeshita E, Miyake O, Ishizuka N. Prediction of lateral pelvic node involvement in low rectal cancer by conventional computed tomography. *Br J Surg* 2007; **94**: 1014-1019

15 **Pomerri F**, Maretto I, Pucciarelli S, Rugge M, Burzi S, Zandonà M, Ambrosi A, Urso E, Muzzio PC, Nitti D. Prediction of rectal lymph node metastasis by pelvic computed tomography measurement. *Eur J Surg Oncol* 2009; **35**: 168-173

16 **Cui CY**, Li L, Liu LZ. [Value of multislice spiral CT in preoperative staging of rectal carcinoma] *Aizheng* 2008; **27**: 196-200

17 **Kanamoto T**, Matsuki M, Okuda J, Inada Y, Tatsugami F, Tanikake M, Yoshikawa S, Narabayashi I, Kawasaki H, Tanaka K, Yamamoto T, Tanigawa N, Egashira Y, Shibayama Y. Preoperative evaluation of local invasion and metastatic lymph nodes of colorectal cancer and mesenteric vascular variations using multidetector-row computed tomography before laparoscopic surgery. *J Comput Assist Tomogr* 2007; **31**: 831-839

18 **Burton S**, Brown G, Bees N, Norman A, Biedrzycki O, Arnaout A, Abulafi AM, Swift RI. Accuracy of CT prediction of poor prognostic features in colonic cancer. *Br J Radiol* 2008; **81**: 10-19

19 **Kirke R**, Rajesh A, Verma R, Bankart MJ. Rectal cancer: incidence of pulmonary metastases on thoracic CT and correlation with T staging. *J Comput Assist Tomogr* 2007; **31**: 569-571

20 **Van Cutsem EJ**, Kataja VV. ESMO Minimum Clinical

Recommendations for diagnosis, adjuvant treatment and follow-up of colon cancer. *Ann Oncol* 2005; **16** Suppl 1: i16-i17
21 **Van Cutsem EJ**, Oliveira J, Kataja VV. ESMO Minimum Clinical Recommendations for diagnosis, treatment and follow-up of advanced colorectal cancer. *Ann Oncol* 2005; **16** Suppl 1: i18-i19
22 **Tveit KM**, Kataja VV. ESMO Minimum Clinical Recommendations for diagnosis, treatment and follow-up of rectal cancer. *Ann Oncol* 2005; **16** Suppl 1: i20-i21
23 **Beets-Tan RG**, Beets GL. Rectal cancer: review with emphasis on MR imaging. *Radiology* 2004; **232**: 335-346
24 **Brown G**. Thin section MRI in multidisciplinary pre-operative decision making for patients with rectal cancer. *Br J Radiol* 2005; **78** Spec No 2: S117-S127
25 **McMahon CJ**, Smith MP. Magnetic resonance imaging in locoregional staging of rectal adenocarcinoma. *Semin Ultrasound CT MR* 2008; **29**: 433-453
26 **Brown G**, Radcliffe AG, Newcombe RG, Dallimore NS, Bourne MW, Williams GT. Preoperative assessment of prognostic factors in rectal cancer using high-resolution magnetic resonance imaging. *Br J Surg* 2003; **90**: 355-364
27 **Kim SH**, Lee JM, Lee MW, Kim GH, Han JK, Choi BI. Diagnostic accuracy of 3.0-Tesla rectal magnetic resonance imaging in preoperative local staging of primary rectal cancer. *Invest Radiol* 2008; **43**: 587-593
28 **Kim CK**, Kim SH, Chun HK, Lee WY, Yun SH, Song SY, Choi D, Lim HK, Kim MJ, Lee J, Lee SJ. Preoperative staging of rectal cancer: accuracy of 3-Tesla magnetic resonance imaging. *Eur Radiol* 2006; **16**: 972-980
29 **Bianchi PP**, Ceriani C, Rottoli M, Torzilli G, Pompili G, Malesci A, Ferraroni M, Montorsi M. Endoscopic ultrasonography and magnetic resonance in preoperative staging of rectal cancer: comparison with histologic findings. *J Gastrointest Surg* 2005; **9**: 1222-1227; discussion 1227-1228
30 **Wieder HA**, Rosenberg R, Lordick F, Geinitz H, Beer A, Becker K, Woertler K, Dobritz M, Siewert JR, Rummeny EJ, Stollfuss JC. Rectal cancer: MR imaging before neoadjuvant chemotherapy and radiation therapy for prediction of tumor-free circumferential resection margins and long-term survival. *Radiology* 2007; **243**: 744-751
31 **Smith NJ**, Shihab O, Arnaout A, Swift RI, Brown G. MRI for detection of extramural vascular invasion in rectal cancer. *AJR Am J Roentgenol* 2008; **191**: 1517-1522
32 **Smith NJ**, Barbachano Y, Norman AR, Swift RI, Abulafi AM, Brown G. Prognostic significance of magnetic resonance imaging-detected extramural vascular invasion in rectal cancer. *Br J Surg* 2008; **95**: 229-236
33 **MERCURY Study Group**. Extramural depth of tumor invasion at thin-section MR in patients with rectal cancer: results of the MERCURY study. *Radiology* 2007; **243**: 132-139

**S- Editor** Tian L **L- Editor** Wang XL **E- Editor** Zheng XM

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.15.2145

*World J Gastroenterol* 2009 May 7; 15(17): 2145-2150
***World Journal of Gastroenterology*** ISSN 1007-9327




# 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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Supported by A special fund for Key University Laboratories from Department of Education of Liaoning Province, No. 2008S233
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Received: December 9, 2008 Revised: March 27, 2009
Accepted: April 3, 2009
Published online: May 7, 2009

## 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%, $\chi^2$ = 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 ($\chi^2$ = 4.400, 6.122 and 11.190, respectively, $P$ < 0.05). The expression of ki-67 was related to the Borrmann's classification ($\chi^2$ = 13.380, $P$ < 0.05). Bcl-2 expression was correlated with the Lauren's classification ($\chi^2$ = 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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Zhao J, Luo XD, Da CL, Xin Y. Clinicopathological significance of B-cell-specific Moloney murine leukemia virus insertion site 1 expression in gastric carcinoma and its precancerous lesion. *World J Gastroenterol* 2009; 15(17): 2145-2150 Available from: URL: http://www.wjgnet.com/1007-9327/15/2145.asp DOI: http://dx.doi.org/10.3748/wjg.15.2145

## 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 | | | $\chi^2$ | *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.

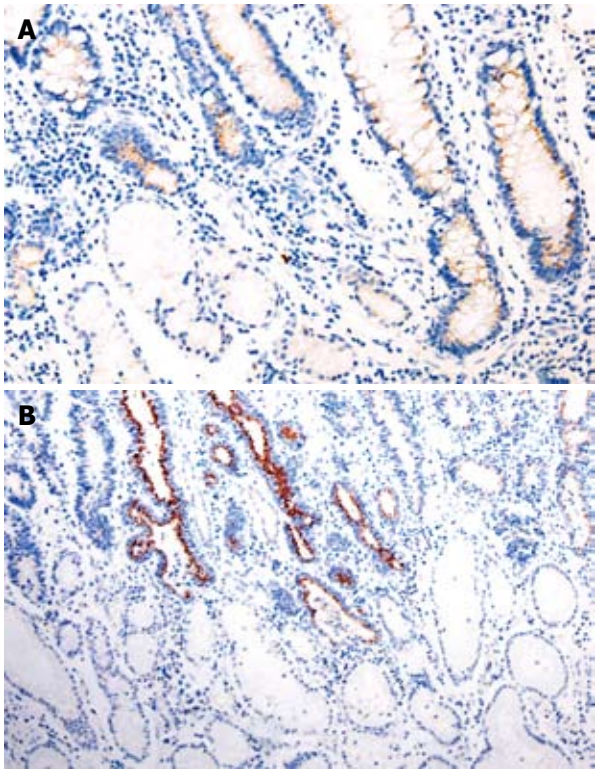


**Figure 1 Expression of Bmi-1 in IM (A, × 200) and GC (B, × 100) (PV-9000).**

### Statistical analysis

Statistical analysis was performed using SPSS 11.5. $\chi^2$ 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 ($\chi^2$ = 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 | | | $\chi^2$ | *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.

($\chi^2$ = 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 ($\chi^2$ = 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

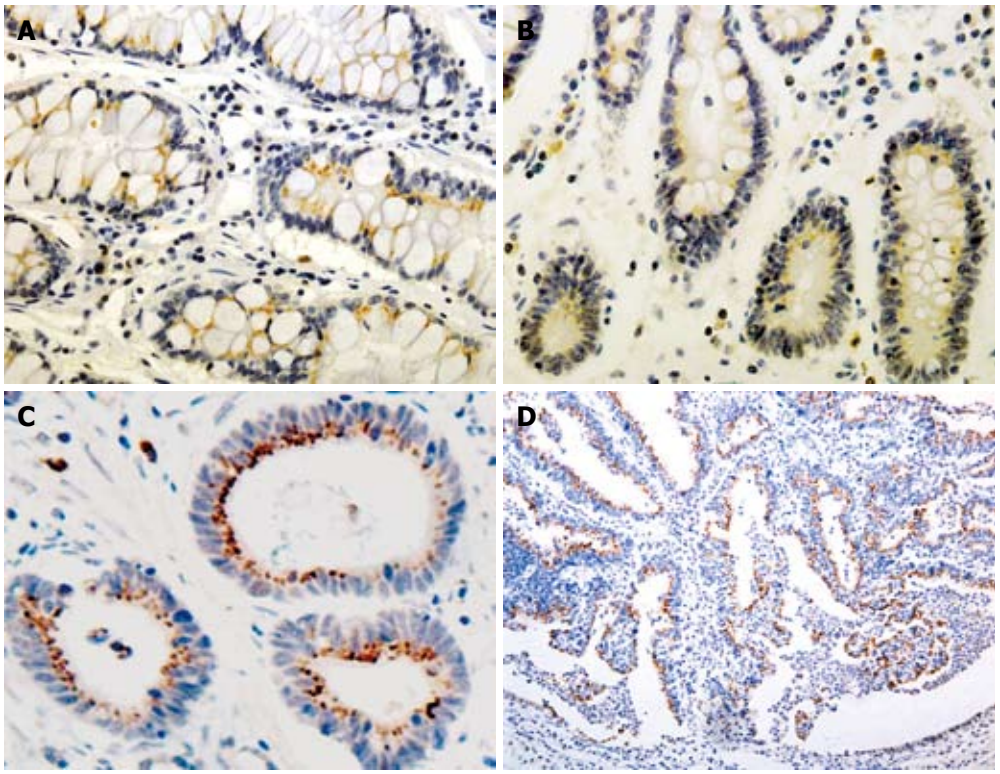


**Figure 2 Expression of Bmi-1 in IM (A), mild DYS (B), gastric tubular adenocarcinoma (C) and papillary adenocarcinoma (D) (PV-9000 A-C × 400, D × 200).**

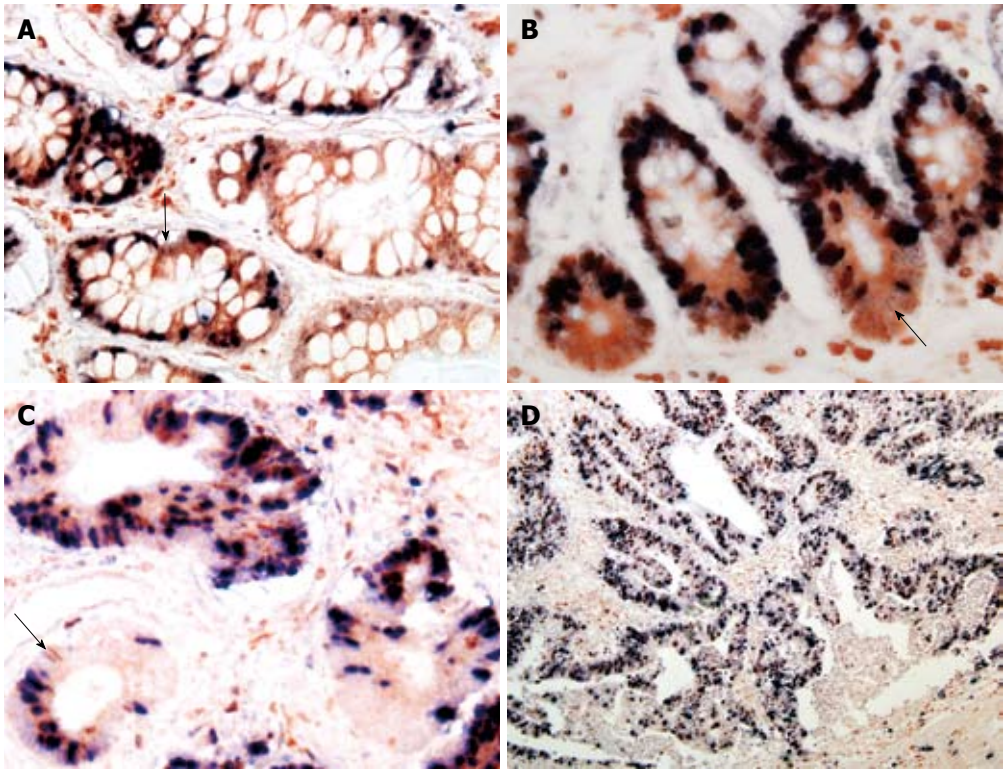


**Figure 3 Distribution of Bcl-2$^+$/ki-67$^-$ cells in IM (A), mild DYS (B), gastric tubular adenocarcinoma (C) and papillary adenocarcinoma (D).** Red fine granules in cytoplasm and unstained nuclei were defined as Bcl-2$^+$/ki-67$^-$ cells as shown by the arrows (Immunohistochemical double staining,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

## REFERENCES


1 **Park IK**, Morrison SJ, Clarke MF. Bmi1, stem cells, and senescence regulation. *J Clin Invest* 2004; **113**: 175-179

2 **Wang H**, Pan K, Zhang HK, Weng DS, Zhou J, Li JJ, Huang W, Song HF, Chen MS, Xia JC. Increased polycomb-group oncogene Bmi-1 expression correlates with poor prognosis in hepatocellular carcinoma. *J Cancer Res Clin Oncol* 2008; **134**: 535-541

3 **Choi YJ**, Choi YL, Cho EY, Shin YK, Sung KW, Hwang YK, Lee SJ, Kong G, Lee JE, Kim JS, Kim JH, Yang JH, Nam SJ. Expression of Bmi-1 protein in tumor tissues is associated with favorable prognosis in breast cancer patients. *Breast Cancer Res Treat* 2009; **113**: 83-93

4 **Reinisch C**, Kandutsch S, Uthman A, Pammer J. BMI-1: a protein expressed in stem cells, specialized cells and tumors of the gastrointestinal tract. *Histol Histopathol* 2006; **21**: 1143-1149

5 **Jacobs JJ**, Kieboom K, Marino S, DePinho RA, van Lohuizen M. The oncogene and Polycomb-group gene bmi-1 regulates cell proliferation and senescence through the ink4a locus. *Nature* 1999; **397**: 164-168

6 **Tateishi K**, Ohta M, Kanai F, Guleng B, Tanaka Y, Asaoka Y, Tada M, Seto M, Jazag A, Lianjie L, Okamoto M, Isayama H, Tada M, Yoshida H, Kawabe T, Omata M. Dysregulated expression of stem cell factor Bmi1 in precancerous lesions of the gastrointestinal tract. *Clin Cancer Res* 2006; **12**: 6960-6966

7 **Jacobs JJ**, Scheijen B, Voncken JW, Kieboom K, Berns A, van Lohuizen M. Bmi-1 collaborates with c-Myc in tumorigenesis by inhibiting c-Myc-induced apoptosis via INK4a/ARF. *Genes Dev* 1999; **13**: 2678-2690

8 **Liu JH**, Song LB, Zhang X, Guo BH, Feng Y, Li XX, Liao WT, Zeng MS, Huang KH. Bmi-1 expression predicts prognosis for patients with gastric carcinoma. *J Surg Oncol* 2008; **97**: 267-272

9 **Lessard J**, Sauvageau G. Bmi-1 determines the proliferative capacity of normal and leukaemic stem cells. *Nature* 2003; **423**: 255-260

10 **Häyry V**, Tanner M, Blom T, Tynninen O, Roselli A, Ollikainen M, Sariola H, Wartiovaara K, Nupponen NN. Copy number alterations of the polycomb gene BMI1 in gliomas. *Acta Neuropathol* 2008; **116**: 97-102

11 **Arnes JB**, Collett K, Akslen LA. Independent prognostic value of the basal-like phenotype of breast cancer and associations with EGFR and candidate stem cell marker BMI-1. *Histopathology* 2008; **52**: 370-380

12 **Dovey JS**, Zacharek SJ, Kim CF, Lees JA. Bmi1 is critical for lung tumorigenesis and bronchioalveolar stem cell expansion. *Proc Natl Acad Sci USA* 2008; **105**: 11857-11862

13 **Molofsky AV**, He S, Bydon M, Morrison SJ, Pardal R. Bmi-1 promotes neural stem cell self-renewal and neural development but not mouse growth and survival by repressing the p16Ink4a and p19Arf senescence pathways. *Genes Dev* 2005; **19**: 1432-1437

14 **Sangiorgi E**, Capecchi MR. Bmi1 is expressed in vivo in intestinal stem cells. *Nat Genet* 2008; **40**: 915-920


**S- Editor** Li LF **L- Editor** Wang XL **E- Editor** Zheng XM

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.15.2151

*World J Gastroenterol* 2009 May 7; 15(17): 2151-2155
***World Journal of Gastroenterology*** ISSN 1007-9327


*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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Received: September 21, 2008 Revised: December 18, 2008
Accepted: December 25, 2008
Published online: May 7, 2009

## 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

**Peer reviewer:** Osman C Ozdogan, Associate Professor, Department of Gastroenterology, Liver Unit, Marmara University School of Medicine, Istanbul 34662, Turkey

Ding SH, Liu J, Wang JP. Efficacy of β-adrenergic blocker plus 5-isosorbide mononitrate and endoscopic band ligation for prophylaxis of esophageal variceal rebleeding: A meta-analysis. *World J Gastroenterol* 2009; 15(17): 2151-2155 Available from: URL: http://www.wjgnet.com/1007-9327/15/2151.asp DOI: http://dx.doi.org/10.3748/wjg.15.2151

## 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 $\chi^2$ 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).

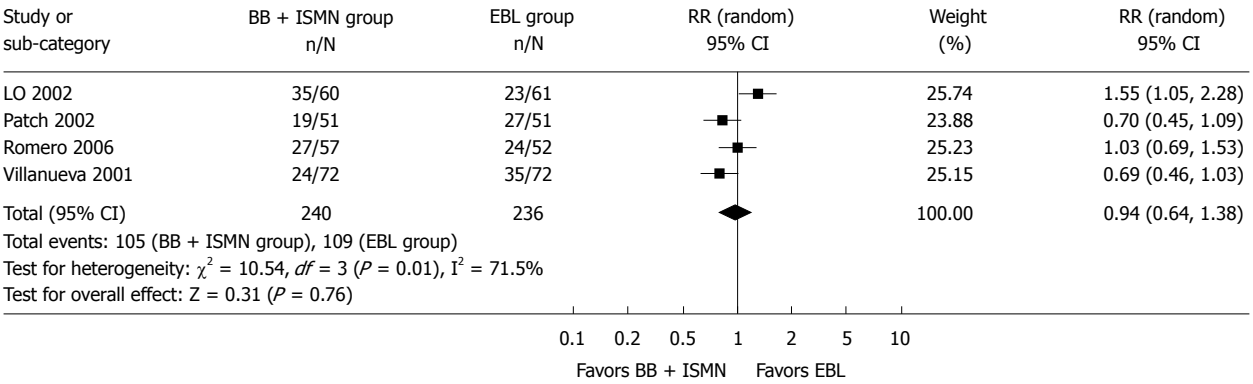
**Bleeding-related deaths:** Three trials evaluated bleeding-related deaths. There was no significant heterogeneity among studies ($P = 0.58$) and no significant difference in the rate of bleeding-related deaths between the BB + ISMN and EBL groups (RR, 0.76; 95% CI: 0.31-1.42; $P = 0.40$) (Figure 1C).

**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

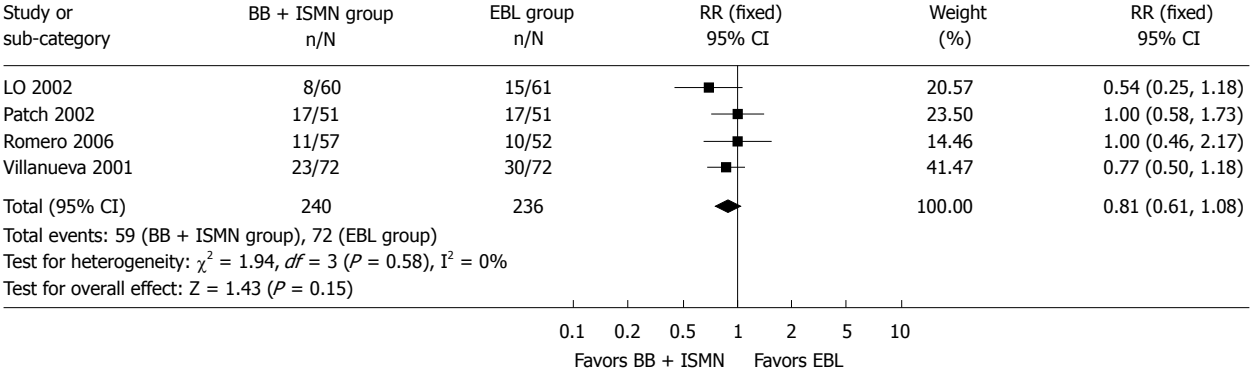
**Table 1 Patient characteristics and Jadad score of included trials**

| 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 |

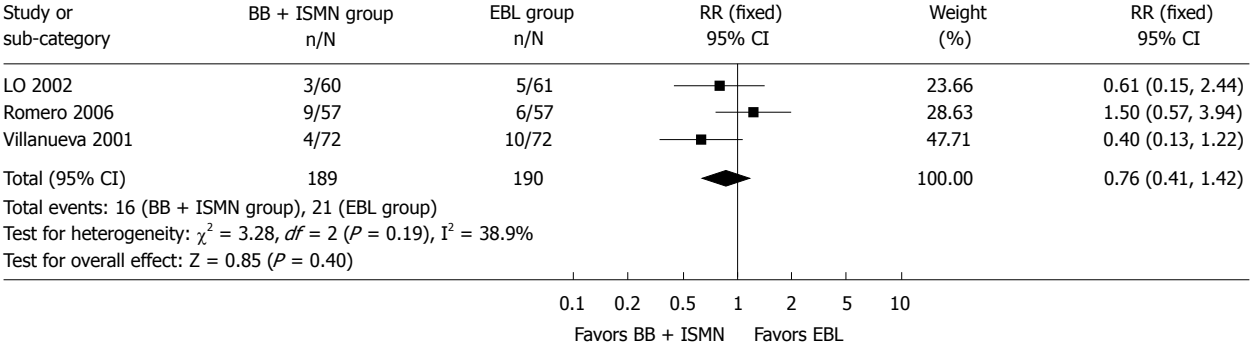
**A** Review: Prophylaxis of esophageal variceal rebleeding
Comparison: 01 BB + ISMN group *vs* EBL group
Outcome: 01 rebleeding rate




**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



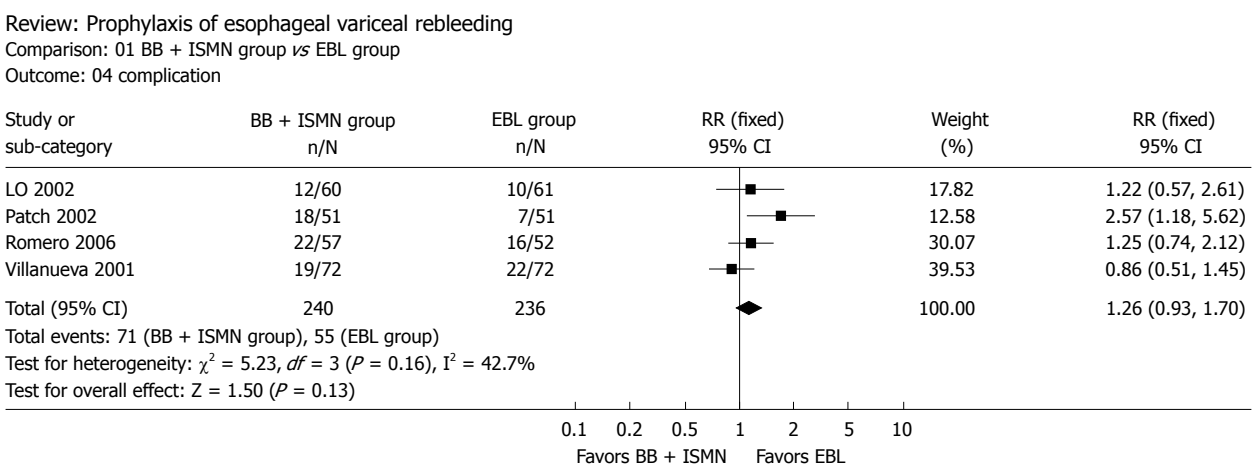



**Figure 1 Comparison between BB + ISMN and EBL.** A: Rebleeding rate; B: All-cause mortality; C: Bleeding-related mortality; D: Complication rate.

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.

## REFERENCES


1 **Bosch J**, Abraldes JG, Groszmann R. Current management of portal hypertension. *J Hepatol* 2003; **38** Suppl 1: S54-S68

2 **Bernard B**, Lebrec D, Mathurin P, Opolon P, Poynard T. Beta-adrenergic antagonists in the prevention of gastrointestinal rebleeding in patients with cirrhosis: a meta-analysis. *Hepatology* 1997; **25**: 63-70

3 **Garcia-Tsao G**, Grace ND, Groszmann RJ, Conn HO, Bermann MM, Patrick MJ, Morse SS, Alberts JL. Short-term effects of propranolol on portal venous pressure. *Hepatology* 1986; **6**: 101-106

4 **Gournay J**, Masliah C, Martin T, Perrin D, Galmiche JP. Isosorbide mononitrate and propranolol compared with propranolol alone for the prevention of variceal rebleeding. *Hepatology* 2000; **31**: 1239-1245

5 **Zhang Q**, Yuan R, Wang H. [The randomized controlled trial of isosorbide mononitrate plus propranolol compared with propranolol alone for the prevention of variceal rebleeding] *Zhonghua Yixue Zazhi* 2002; **82**: 1157-1159

6 **Villanueva C**, Miñana J, Ortiz J, Gallego A, Soriano G, Torras X, Sáinz S, Boadas J, Cussó X, Guarner C, Balanzó J. Endoscopic ligation compared with combined treatment with nadolol and isosorbide mononitrate to prevent recurrent variceal bleeding. *N Engl J Med* 2001; **345**: 647-655

7 **Lo GH**, Chen WC, Chen MH, Hsu PI, Lin CK, Tsai WL, Lai KH. Banding ligation versus nadolol and isosorbide mononitrate for the prevention of esophageal variceal rebleeding. *Gastroenterology* 2002; **123**: 728-734

8 **Patch D**, Sabin CA, Goulis J, Gerunda G, Greenslade L, Merkel C, Burroughs AK. A randomized, controlled trial of medical therapy versus endoscopic ligation for the prevention of variceal rebleeding in patients with cirrhosis. *Gastroenterology* 2002; **123**: 1013-1019

9 **Romero G**, Kravetz D, Argonz J, Vulcano C, Suarez A, Fassio E, Dominguez N, Bosco A, Muñoz A, Salgado P, Terg R. Comparative study between nadolol and 5-isosorbide mononitrate vs. endoscopic band ligation plus sclerotherapy in the prevention of variceal rebleeding in cirrhotic patients: a randomized controlled trial. *Aliment Pharmacol Ther* 2006; **24**: 601-611

10 **Moher D**, Pham B, Jones A, Cook DJ, Jadad AR, Moher M, Tugwell P, Klassen TP. Does quality of reports of randomised trials affect estimates of intervention efficacy reported in meta-analyses? *Lancet* 1998; **352**: 609-613

11 **Garcia-Tsao G**, Sanyal AJ, Grace ND, Carey W. Prevention and management of gastroesophageal varices and variceal hemorrhage in cirrhosis. *Hepatology* 2007; **46**: 922-938

12 **Karsan HA**, Morton SC, Shekelle PG, Spiegel BM, Suttorp MJ, Edelstein MA, Gralnek IM. Combination endoscopic band ligation and sclerotherapy compared with endoscopic band ligation alone for the secondary prophylaxis of esophageal variceal hemorrhage: a meta-analysis. *Dig Dis Sci* 2005; **50**: 399-406

13 **García-Pagán JC**, Morillas R, Bañares R, Albillos A, Villanueva C, Vila C, Genescà J, Jimenez M, Rodriguez M, Calleja JL, Balanzó J, García-Durán F, Planas R, Bosch J. Propranolol plus placebo versus propranolol plus isosorbide-5-mononitrate in the prevention of a first variceal bleed: a double-blind RCT. *Hepatology* 2003; **37**: 1260-1266

14 **de la Peña J**, Brullet E, Sanchez-Hernández E, Rivero M, Vergara M, Martin-Lorente JL, Garcia Suárez C. Variceal ligation plus nadolol compared with ligation for prophylaxis of variceal rebleeding: a multicenter trial. *Hepatology* 2005; **41**: 572-578

15 **Lo GH**, Chen WC, Chen MH, Lin CP, Lo CC, Hsu PI, Cheng JS, Lai KH. Endoscopic ligation vs. nadolol in the prevention of first variceal bleeding in patients with cirrhosis. *Gastrointest Endosc* 2004; **59**: 333-338


**S- Editor** Cheng JX **L- Editor** Ma JY **E- Editor** Zheng XM

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.15.2156

*World J Gastroenterol* 2009 May 7; 15(17): 2156-2161
***World Journal of Gastroenterology*** ISSN 1007-9327


*CASE REPORT*

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

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Received: November 14, 2008 Revised: March 24, 2009
Accepted: March 31, 2009
Published online: May 7, 2009

## 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

**Peer reviewer:** Giovanni Maconi, MD, Department of Gastroenterology, 'L.Sacco' University Hospital, Via G.B. Grassi, 74, Milan 20157, Italy

Sheikh RA, Prindiville TP, Pecha RE, Ruebner BH. Unusual presentations of eosinophilic gastroenteritis: Case series and review of literature. *World J Gastroenterol* 2009; 15(17): 2156-2161 Available from: URL: http://www.wjgnet.com/1007-9327/15/2156.asp DOI: http://dx.doi.org/10.3748/wjg.15.2156

## 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

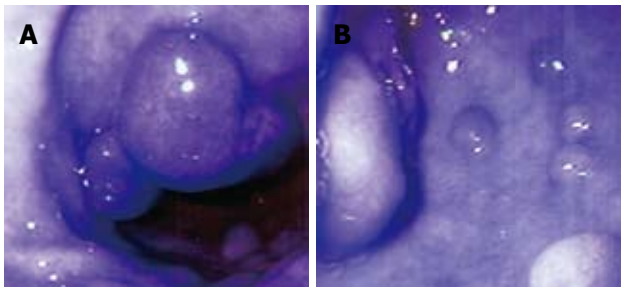


**Figure 1 Endoscopic appearance of stomach (A and B) showing multiple gastric antral polyps of 4-10 mm in size and antral mucosal erythema.**

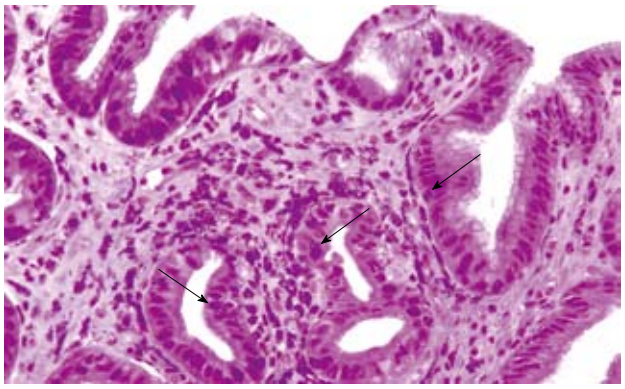

**Figure 2 Histological appearance of the gastric polyp showing eosinophilic infiltration of the lamina propria by numerous degranulated eosinophils and some polymorphonuclear cells (arrows).** Some intraepithelial eosinophils are also seen. (HE, × 200).

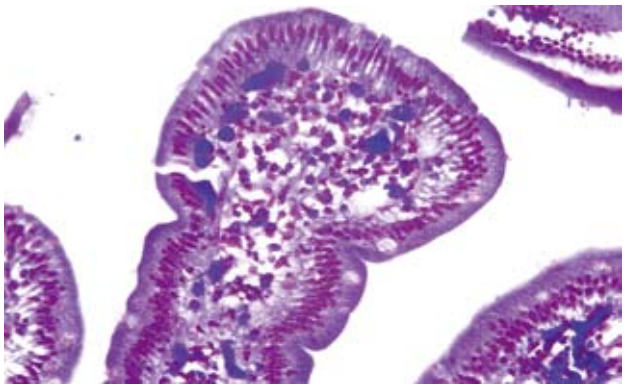

**Figure 3 Histological appearance of duodenal mucosal biopsy demonstrating moderately severe infiltration with eosinophils and some intraepithelial eosinophils.** (HE, × 200).

**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.

## REFERENCES


1 **Daneshjoo R**, J Talley N. Eosinophilic gastroenteritis. *Curr Gastroenterol Rep* 2002; **4**: 366-372

2 **Khan S**, Orenstein SR. Eosinophilic gastroenteritis. *Gastroenterol Clin North Am* 2008; **37**: 333-348, v

3 **Yun MY**, Cho YU, Park IS, Choi SK, Kim SJ, Shin SH, Kim KR. Eosinophilic gastroenteritis presenting as small bowel obstruction: a case report and review of the literature. *World J Gastroenterol* 2007; **13**: 1758-1760

4 **Chen MJ**, Chu CH, Lin SC, Shih SC, Wang TE. Eosinophilic gastroenteritis: clinical experience with 15 patients. *World J Gastroenterol* 2003; **9**: 2813-2816

5 **Khan S**. Eosinophilic gastroenteritis. *Best Pract Res Clin Gastroenterol* 2005; **19**: 177-198

6 **Kaijser R**. Zur Kenntnis der allergischen Affektionen des Verdawungskanal vom standpunkt desi Chirugen aus. *Arch Klin Chir* 1937; **188**: 36-64

7 **Talley NJ**, Shorter RG, Phillips SF, Zinsmeister AR. Eosinophilic gastroenteritis: a clinicopathological study of patients with disease of the mucosa, muscle layer, and subserosal tissues. *Gut* 1990; **31**: 54-58

8 **Naylor AR**. Eosinophilic gastroenteritis. *Scott Med J* 1990; **35**: 163-165

9 **Lee M**, Hodges WG, Huggins TL, Lee EL. Eosinophilic gastroenteritis. *South Med J* 1996; **89**: 189-194

10 **Rankin SM**, Conroy DM, Williams TJ. Eotaxin and eosinophil recruitment: implications for human disease. *Mol Med Today* 2000; **6**: 20-27
11 **Kelly KJ**. Eosinophilic gastroenteritis. *J Pediatr Gastroenterol Nutr* 2000; **30** Suppl: S28-S35
12 **Neustrom MR**, Friesen C. Treatment of eosinophilic gastroenteritis with montelukast. *J Allergy Clin Immunol* 1999; **104**: 506
13 **Drumm B**, Rhoads JM, Stringer DA, Sherman PM, Ellis LE, Durie PR. Peptic ulcer disease in children: etiology, clinical findings, and clinical course. *Pediatrics* 1988; **82**: 410-414
14 **Weller PF**. The immunobiology of eosinophils. *N Engl J Med* 1991; **324**: 1110-1118
15 **Winter HS**, Madara JL, Stafford RJ, Grand RJ, Quinlan JE, Goldman H. Intraepithelial eosinophils: a new diagnostic criterion for reflux esophagitis. *Gastroenterology* 1982; **83**: 818-823
16 **Garcia-Zepeda EA**, Rothenberg ME, Ownbey RT, Celestin J, Leder P, Luster AD. Human eotaxin is a specific chemoattractant for eosinophil cells and provides a new mechanism to explain tissue eosinophilia. *Nat Med* 1996; **2**: 449-456
17 **Walker NI**, Croese J, Clouston AD, Parry M, Loukas A, Prociv P. Eosinophilic enteritis in northeastern Australia. Pathology, association with Ancylostoma caninum, and implications. *Am J Surg Pathol* 1995; **19**: 328-337
18 **Croese J**, Loukas A, Opdebeeck J, Prociv P. Occult enteric infection by Ancylostoma caninum: a previously unrecognized zoonosis. *Gastroenterology* 1994; **106**: 3-12
19 **Michet CJ Jr**, Rakela J, Luthra HS. Auranofin-associated colitis and eosinophilia. *Mayo Clin Proc* 1987; **62**: 142-144
20 **Anttila VJ**, Valtonen M. Carbamazepine-induced eosinophilic colitis. *Epilepsia* 1992; **33**: 119-121
21 **Klein NC**, Hargrove RL, Sleisenger MH, Jeffries GH. Eosinophilic gastroenteritis. *Medicine* (Baltimore) 1970; **49**: 299-319
22 **Scudamore HH**, Phillips SF, Swedlund HA, Gleich GJ. Food allergy manifested by eosinophilia, elevated immunoglobulin E level, and protein-losing enteropathy: the syndrome of allergic gastroenteropathy. *J Allergy Clin Immunol* 1982; **70**: 129-138
23 **Chowdhury A**, Dhali GK, Banerjee PK. Etiology of gastric outlet obstruction. *Am J Gastroenterol* 1996; **91**: 1679
24 **Hummer-Ehret BH**, Rohrschneider WK, Oleszczuk-Raschke K, Darge K, Nutzenadel W, Troger J. Eosinophilic gastroenteritis mimicking idiopathic hypertrophic pyloric stenosis. *Pediatr Radiol* 1998; **28**: 711-713
25 **Karande T**, Oak SN, Trivedi A, Karmarkar S, Kulkarni B, Kalgutkar A. Proximal jejunal obstruction due to eosinophilic gastroenteritis. *J Postgrad Med* 1996; **42**: 121-123
26 **Wig JD**, Goenka MK, Bhasin DK, Vaiphei K. Eosinophilic gastroenteritis presenting as acute intestinal obstruction. *Indian J Gastroenterol* 1995; **14**: 104-105
27 **Matsushita M**, Hajiro K, Morita Y, Takakuwa H, Suzaki T. Eosinophilic gastroenteritis involving the entire digestive tract. *Am J Gastroenterol* 1995; **90**: 1868-1870
28 **Jacobson LB**. Diffuse eosinophilic gastroenteritis: an adult form of allergic gastroenteropathy. Report of a case with probable protein-losing enteropathy. *Am J Gastroenterol* 1970; **54**: 580-588
29 **Schoonbroodt D**, Horsmans Y, Laka A, Geubel AP, Hoang P. Eosinophilic gastroenteritis presenting with colitis and cholangitis. *Dig Dis Sci* 1995; **40**: 308-314
30 **Redondo Cerezo E**, Moreno Platero JJ, Garcia Dominguez E, Gonzalez Aranda Y, Cabello Tapia MJ, Martinez Tirado P, Lopez de Hierro Ruiz ML, Gomez Garcia M. [Gastroenteritis eosinophilic presenting as colitis with acute abdomen] *Gastroenterol Hepatol* 2000; **23**: 477-479
31 **Box JC**, Tucker J, Watne AL, Lucas G. Eosinophilic colitis presenting as a left-sided colocolonic intussusception with secondary large bowel obstruction: an uncommon entity with a rare presentation. *Am Surg* 1997; **63**: 741-743
32 **Blanco-Guerra C**, Cazana JL, Villas F, Bazire P, Martinez F. Ileal perforation due to eosinophilic gastroenteritis. *Am J Gastroenterol* 1991; **86**: 1689-1690
33 **Maeshima A**, Murakami H, Sadakata H, Saitoh T, Matsushima T, Tamura J, Karasawa M, Naruse T. Eosinophilic gastroenteritis presenting with acute pancreatitis. *J Med* 1997; **28**: 265-272
34 **Euscher E**, Vaswani K, Frankel W. Eosinophilic pancreatitis: a rare entity that can mimic a pancreatic neoplasm. *Ann Diagn Pathol* 2000; **4**: 379-385
35 **Gregg JA**, Utz DC. Eosinophilic cystitis associated with eosinophilic gastroenteritis. *Mayo Clin Proc* 1974; **49**: 185-187
36 **Robert F**, Omura E, Durant JR. Mucosal eosinophilic gastroenteritis with systemic involvement. *Am J Med* 1977; **62**: 139-143
37 **Cello JP**. Eosinophilic gastroenteritis--a complex disease entity. *Am J Med* 1979; **67**: 1097-1104
38 **Di Gioacchino M**, Pizzicannella G, Fini N, Falasca F, Antinucci R, Masci S, Mezzetti A, Marzio L, Cuccurullo F. Sodium cromoglycate in the treatment of eosinophilic gastroenteritis. *Allergy* 1990; **45**: 161-166
39 **Perez-Millan A**, Martin-Lorente JL, Lopez-Morante A, Yuguero L, Saez-Royuela F. Subserosal eosinophilic gastroenteritis treated efficaciously with sodium cromoglycate. *Dig Dis Sci* 1997; **42**: 342-344
40 **Melamed I**, Feanny SJ, Sherman PM, Roifman CM. Benefit of ketotifen in patients with eosinophilic gastroenteritis. *Am J Med* 1991; **90**: 310-314
41 **Shirai T**, Hashimoto D, Suzuki K, Osawa S, Aonahata M, Chida K, Nakamura H. Successful treatment of eosinophilic gastroenteritis with suplatast tosilate. *J Allergy Clin Immunol* 2001; **107**: 924-925
42 **Garrett JK**, Jameson SC, Thomson B, Collins MH, Wagoner LE, Freese DK, Beck LA, Boyce JA, Filipovich AH, Villanueva JM, Sutton SA, Assa'ad AH, Rothenberg ME. Anti-interleukin-5 (mepolizumab) therapy for hypereosinophilic syndromes. *J Allergy Clin Immunol* 2004; **113**: 115-119
43 **Felt-Bersma RJ**, Meuwissen SG, van Velzen D. Perforation of the small intestine due to eosinophilic gastroenteritis. *Am J Gastroenterol* 1984; **79**: 442-445
44 **Martin A**, Castagliuolo I, Floreani A, De Lazzari F, Sturniolo GC, Di Mario F, Del Favero G, Mastropaolo G, Naccarato R. Eosinophilic gastroenteritis: report of two atypical cases. *Ital J Gastroenterol* 1991; **23**: 81-85


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

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.15.2162

*World J Gastroenterol* 2009 May 7; 15(17): 2162-2165
***World Journal of Gastroenterology*** ISSN 1007-9327


*CASE REPORT*

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

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Received: January 11, 2009 Revised: March 14, 2009
Accepted: March 21, 2009
Published online: May 7, 2009

## 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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Akahoshi K, Motomura Y, Kubokawa M, Matsui N, Oda M, Okamoto R, Endo S, Higuchi N, Kashiwabara Y, Oya M, Akahane H, Akiba H. Endoscopic submucosal dissection of a rectal carcinoid tumor using grasping type scissors forceps. *World J Gastroenterol* 2009; 15(17): 2162-2165 Available from: URL: http://www.wjgnet.com/1007-9327/15/2162.asp DOI: http://dx.doi.org/10.3748/wjg.15.2162

## 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.

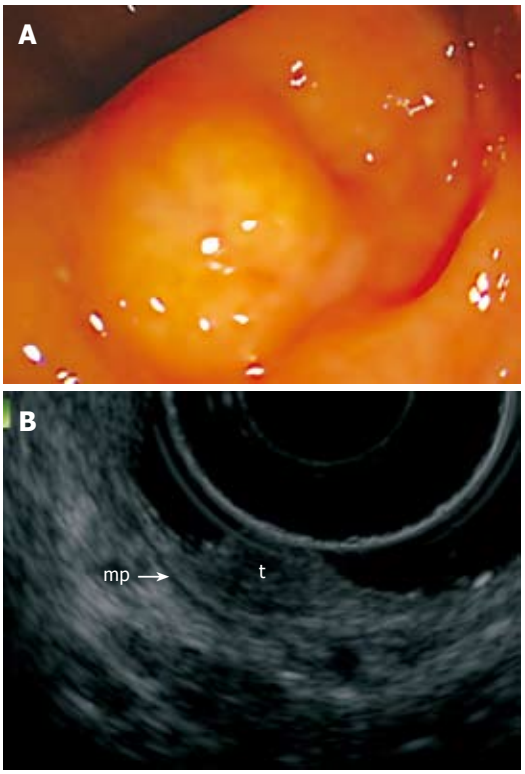


**Figure 1 Pretherapeutic examinations of rectal carcinoid.** A: Endoscopic view of the small rectal carcinoid; B: EUS showing a hypoechoic solid tumor (t) in the superficial submucosa. Arrow-mp: Muscularis propria.

### *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.

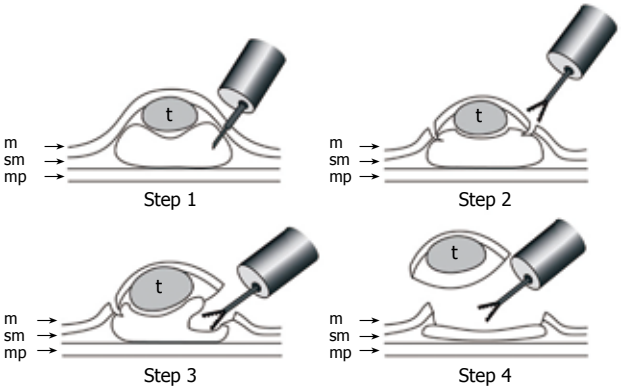


**Figure 3 Schematic shows ESD using GSF.** Step 1: A concentrated glycerin solution mixed with a small volume of epinephrine and indigo carmine dye is injected into the submucosal layer around the target lesion to lift the entire lesion; Step 2: The lesion is separated from the surrounding normal mucosa 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

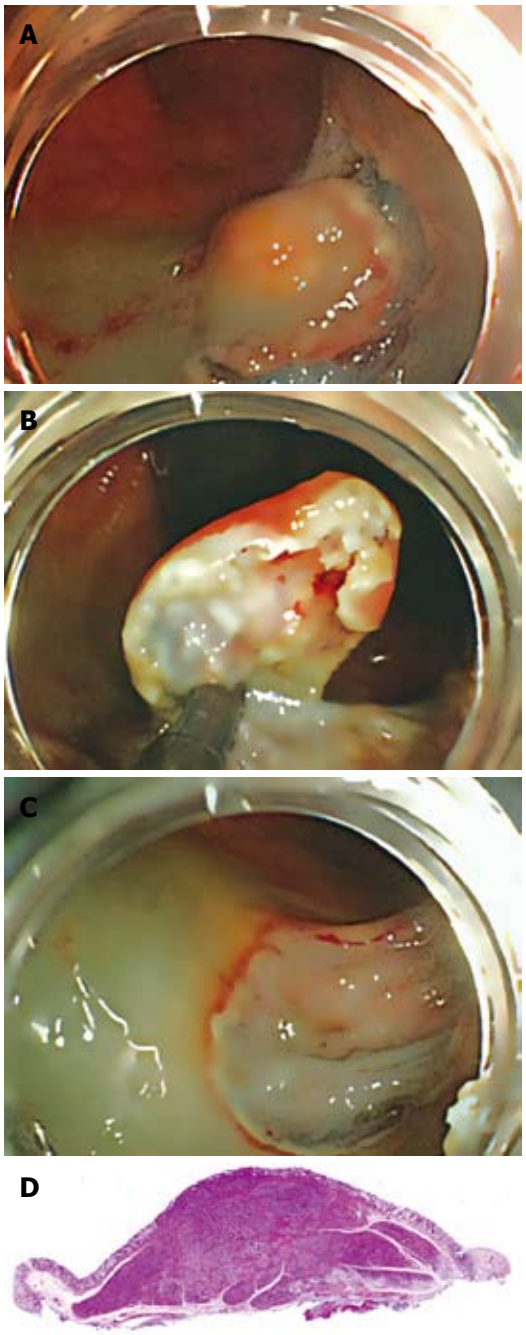


**Figure 4 ESD using GSF of rectal carcinoid.** A: Endoscopic view of the partial circumferential incision of the tumor using GSF; B: Endoscopic view of the submucosal exfoliation under the tumor using GSF; C: The lesion is cut completely from the muscle layer; D: The resected specimen showing curative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

## REFERENCES


1 **Soga J**. Carcinoids of the rectum: an evaluation of 1271 reported cases. *Surg Today* 1997; **27**: 112-119
2 **Stinner B**, Kisker O, Zielke A, Rothmund M. Surgical management for carcinoid tumors of small bowel, appendix, colon, and rectum. *World J Surg* 1996; **20**: 183-188
3 **Fujishiro M**. Perspective on the practical indications of endoscopic submucosal dissection of gastrointestinal neoplasms. *World J Gastroenterol* 2008; **14**: 4289-4295
4 **Abe T**, Kakemura T, Fujinuma S, Maetani I. Successful outcomes of EMR-L with 3D-EUS for rectal carcinoids compared with historical controls. *World J Gastroenterol* 2008; **14**: 4054-4058
5 **Ono A**, Fujii T, Saito Y, Matsuda T, Lee DT, Gotoda T, Saito D. Endoscopic submucosal resection of rectal carcinoid tumors with a ligation device. *Gastrointest Endosc* 2003; **57**: 583-587
6 **Onozato Y**, Kakizaki S, Ishihara H, Iizuka H, Sohara N, Okamura S, Mori M, Itoh H. Endoscopic submucosal dissection for rectal tumors. *Endoscopy* 2007; **39**: 423-427
7 **Ono H**, Kondo H, Gotoda T, Shirao K, Yamaguchi H, Saito D, Hosokawa K, Shimoda T, Yoshida S. Endoscopic mucosal resection for treatment of early gastric cancer. *Gut* 2001; **48**: 225-229
8 **Fujishiro M**. Endoscopic submucosal dissection for stomach neoplasms. *World J Gastroenterol* 2006; **12**: 5108-5112
9 **Neuhaus H**, Costamagna G, Deviere J, Fockens P, Ponchon T, Rosch T. Endoscopic submucosal dissection (ESD) of early neoplastic gastric lesions using a new double-channel endoscope (the "R-scope"). *Endoscopy* 2006; **38**: 1016-1023
10 **Akahoshi K**, Akahane H, Murata A, Akiba H, Oya M. Endoscopic submucosal dissection using a novel grasping type scissors forceps. *Endoscopy* 2007; **39**: 1103-1105
11 **Akahoshi K**, Honda K, Akahane H, Akiba H, Matsui N, Motomura Y, Kubokawa M, Endo S, Higuchi N, Oya M. Endoscopic submucosal dissection by using a grasping-type scissors forceps: a preliminary clinical study (with video). *Gastrointest Endosc* 2008; **67**: 1128-1133
12 **Ishikawa H**, Imanishi K, Otani T, Okuda S, Tatsuta M, Ishiguro S. Effectiveness of endoscopic treatment of carcinoid tumors of the rectum. *Endoscopy* 1989; **21**: 133-135
13 **Fujimura Y**, Mizuno M, Takeda M, Sato I, Hoshika K, Uchida J, Kihara T, Mure T, Sano K, Moriya T. A carcinoid tumor of the rectum removed by strip biopsy. *Endoscopy* 1993; **25**: 428-430
14 **Imada-Shirakata Y**, Sakai M, Kajiyama T, Kin G, Inoue K, Torii A, Kishimoto H, Ueda S, Okuma M. Endoscopic resection of rectal carcinoid tumors using aspiration lumpectomy. *Endoscopy* 1997; **29**: 34-38
15 **Berkelhammer C**, Jasper I, Kirvaitis E, Schreiber S, Hamilton J, Walloch J. "Band-snare" resection of small rectal carcinoid tumors. *Gastrointest Endosc* 1999; **50**: 582-585
16 **Akahoshi K**, Fujimaru T, Nakanishi K, Harada N, Nawata H. Endosonography probe-guided endoscopic resection of small flat rectal carcinoid tumor using band ligation technique. *Endoscopy* 2001; **33**: 471


**S- Editor** Li LF **L- Editor** Logan S **E- Editor** Ma WH

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.15.2166

*World J Gastroenterol* 2009 May 7; 15(17): 2166-2169
***World Journal of Gastroenterology*** ISSN 1007-9327


*CASE REPORT*

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

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Received: February 16, 2009 Revised: March 23, 2009
Accepted: March 30, 2009
Published online: May 7, 2009

## 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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Chiba M, Sugawara T, Tozawa H, Tsuda H, Abe T, Tokairin T, Ono I, Ushiyama E. Lansoprazole-associated collagenous colitis: Diffuse mucosal cloudiness mimicking ulcerative colitis. *World J Gastroenterol* 2009; 15(17): 2166-2169 Available from: URL: http://www.wjgnet.com/1007-9327/15/2166.asp DOI: http://dx.doi.org/10.3748/wjg.15.2166

## 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

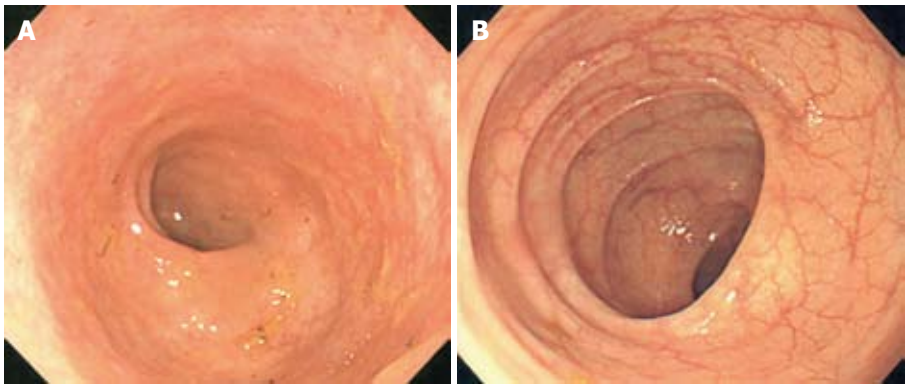


**Figure 1 Colonoscopy on April 16 (A) and May 17 (B), 2007 showed diffuse cloudiness of mucosa in the colon and clear normal vascular patterns, respectively.**

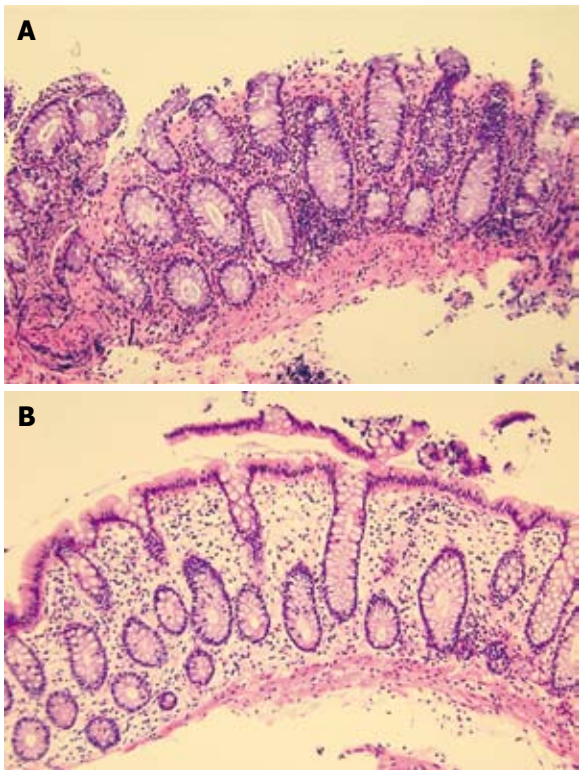


**Figure 2 Biopsy specimens taken on April 16 (A) and May 17 (B), 2007 (hematoxylin and eosin staining, × 100).** The former showed erosion, moderate infiltration of inflammatory cells in the lamina propria, and subepithelial collagenous thickening. The latter showed disappearance of these abnormalities.

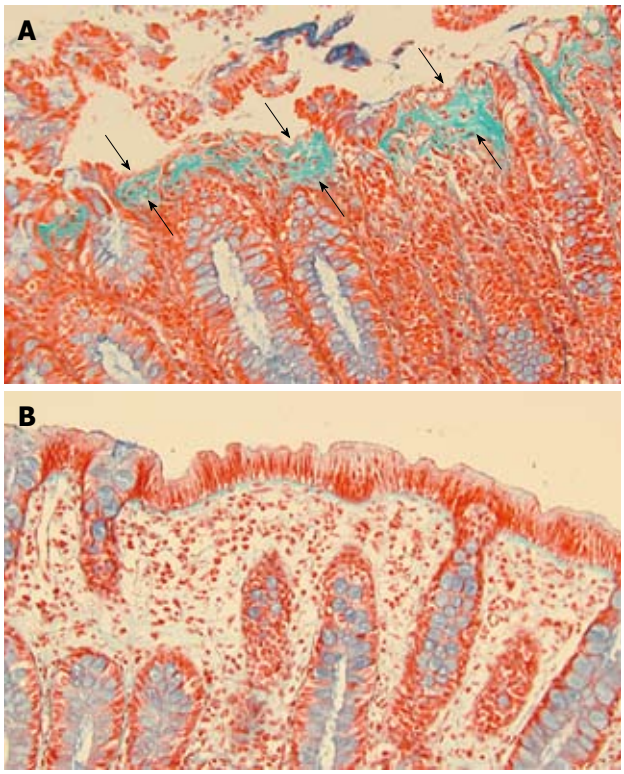


**Figure 3 Biopsy specimens taken on April 16 (A) and May 17 (B), 2007 (Masson's trichrome staining, × 200).** Subepithelial collagenous thickening (A, arrows) disappeared on May 17 (B).

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.

## REFERENCES

1 **Veress B**, Löfberg R, Bergman L. Microscopic colitis syndrome. *Gut* 1995; **36**: 880-886
2 **Pimentel RR**, Achkar E, Bedford R. Collagenous colitis. A treatable disease with an elusive diagnosis. *Dig Dis Sci* 1995; **40**: 1400-1404
3 **Bohr J**, Tysk C, Eriksson S, Abrahamsson H, Järnerot G. Collagenous colitis: a retrospective study of clinical presentation and treatment in 163 patients. *Gut* 1996; **39**: 846-851
4 **Zins BJ**, Sandborn WJ, Tremaine WJ. Collagenous and lymphocytic colitis: subject review and therapeutic alternatives. *Am J Gastroenterol* 1995; **90**: 1394-1400
5 **Riddell RH**, Tanaka M, Mazzoleni G. Non-steroidal anti-inflammatory drugs as a possible cause of collagenous colitis: a case-control study. *Gut* 1992; **33**: 683-686
6 **Kakar S**, Pardi DS, Burgart LJ. Colonic ulcers accompanying collagenous colitis: implication of nonsteroidal anti-inflammatory drugs. *Am J Gastroenterol* 2003; **98**: 1834-1837

7 **Berrebi D**, Sautet A, Flejou JF, Dauge MC, Peuchmaur M, Potet F. Ticlopidine induced colitis: a histopathological study including apoptosis. *J Clin Pathol* 1998; **51**: 280-283
8 **Roubenoff R**, Ratain J, Giardiello F, Hochberg M, Bias W, Lazenby A, Yardley J. Collagenous colitis, enteropathic arthritis, and autoimmune diseases: results of a patient survey. *J Rheumatol* 1989; **16**: 1229-1232
9 **Wilcox GM**, Mattia A. Collagenous colitis associated with lansoprazole. *J Clin Gastroenterol* 2002; **34**: 164-166
10 **Thomson RD**, Lestina LS, Bensen SP, Toor A, Maheshwari Y, Ratcliffe NR. Lansoprazole-associated microscopic colitis: a case series. *Am J Gastroenterol* 2002; **97**: 2908-2913
11 **Rammer M**, Kirchgatterer A, Höbling W, Knoflach P. Lansoprazole-associated collagenous colitis: a case report. *Z Gastroenterol* 2005; **43**: 657-660
12 **Hilmer SN**, Heap TR, Eckstein RP, Lauer CS, Shenfield GM. Microscopic colitis associated with exposure to lansoprazole. *Med J Aust* 2006; **184**: 185-186
13 **Katsinelos P**, Katsos I, Patsiaoura K, Xiarchos P, Goulis I, Eugenidis N. A new endoscopic appearance of collagenous colitis. *Endoscopy* 1997; **29**: 135
14 **Yabe M**, Igarashi K, Hata K, Ho N, Tsukioka S, Shibuya H. A case of collagenous colitis with a unique endoscopic appearance. *Gastroenterol Endosc* 1997; **39**: 1099-1104
15 **Cruz-Correa M**, Milligan F, Giardiello FM, Bayless TM, Torbenson M, Yardley JH, Jackson FW, Wilson Jackson F. Collagenous colitis with mucosal tears on endoscopic insufflation: a unique presentation. *Gut* 2002; **51**: 600
16 **Wickbom A**, Lindqvist M, Bohr J, Ung KA, Bergman J, Eriksson S, Tysk C. Colonic mucosal tears in collagenous colitis. *Scand J Gastroenterol* 2006; **41**: 726-729
17 **Richieri JP**, Bonneau HP, Cano N, Di Costanzo J, Martin J. Collagenous colitis: an unusual endoscopic appearance. *Gastrointest Endosc* 1993; **39**: 192-194
18 **Watanabe T**, Hirakawa K, Sato S, Kochi S, Nakajima Y, Aoyagi K, Matsumoto T, Iida M. A case with collagenous colitis and multiple longitudinal ulcers. *Gastroenterol Endosc* 2008; **50**: 27-33

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

Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.15.2170

*World J Gastroenterol* 2009 May 7; 15(17): 2170-2171
***World Journal of Gastroenterology*** ISSN 1007-9327


*LETTERS TO THE EDITOR*

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

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Received: December 30, 2008 Revised: February 11, 2009
Accepted: February 18, 2009
Published online: May 7, 2009

## 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

**Peer reviewer:** Valentin Fuhrmann, MD, Department of Internal Medicine 4, Intensive Care Unit, Medical University Vienna, Waehringer Guertel 18-20, A-1090 Vienna, Austria

Kapoor S. Emerging clinical and therapeutic applications of *Nigella sativa* in gastroenterology. *World J Gastroenterol* 2009; 15(17): 2170-2171 Available from: URL: http://www.wjgnet.com/1007-9327/15/2170.asp DOI: http://dx.doi.org/10.3748/wjg.15.2170

## 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.

## REFERENCES


1 **Yildiz F**, Coban S, Terzi A, Ates M, Aksoy N, Cakir H, Ocak AR, Bitiren M. Nigella sativa relieves the deleterious effects of ischemia reperfusion injury on liver. *World J Gastroenterol* 2008; **14**: 5204-5209

2 **Al-Johar D**, Shinwari N, Arif J, Al-Sanea N, Jabbar AA, El-Sayed R, Mashhour A, Billedo G, El-Doush I, Al-Saleh I. Role of Nigella sativa and a number of its antioxidant constituents towards azoxymethane-induced genotoxic effects and colon cancer in rats. *Phytother Res* 2008; **22**: 1311-1323

3 **Norwood AA**, Tucci M, Benghuzzi H. A comparison of 5-fluorouracil and natural chemotherapeutic agents, EGCG and thymoquinone, delivered by sustained drug delivery on colon cancer cells. *Biomed Sci Instrum* 2007; **43**: 272-277

4 **Ait Mbarek L**, Ait Mouse H, Elabbadi N, Bensalah M, Gamouh A, Aboufatima R, Benharref A, Chait A, Kamal M, Dalal A, Zyad A. Anti-tumor properties of blackseed (Nigella sativa L.) extracts. *Braz J Med Biol Res* 2007; **40**: 839-847

5 **Iddamaldeniya SS**, Thabrew MI, Wickramasinghe SM, Ratnatunge N, Thammitiyagodage MG. A long-term investigation of the anti-hepatocarcinogenic potential of an indigenous medicine comprised of Nigella sativa, Hemidesmus indicus and Smilax glabra. *J Carcinog* 2006; **5**: 11

6 **Sethi G**, Ahn KS, Aggarwal BB. Targeting nuclear factor-kappa B activation pathway by thymoquinone: role in suppression of antiapoptotic gene products and enhancement of apoptosis. *Mol Cancer Res* 2008; **6**: 1059-1070

7 **Rchid H**, Chevassus H, Nmila R, Guiral C, Petit P, Chokaïri M, Sauvaire Y. Nigella sativa seed extracts enhance glucose-induced insulin release from rat-isolated Langerhans islets. *Fundam Clin Pharmacol* 2004; **18**: 525-529

8 **Meddah B**, Ducroc R, El Abbes Faouzi M, Eto B, Mahraoui L, Benhaddou-Andaloussi A, Martineau LC, Cherrah Y, Haddad PS. Nigella sativa inhibits intestinal glucose absorption and improves glucose tolerance in rats. *J Ethnopharmacol* 2009; **121**: 419-424

9 **Demir H**, Kanter M, Coskun O, Uz YH, Koc A, Yildiz A. Effect of black cumin (Nigella sativa) on heart rate, some hematological values, and pancreatic beta-cell damage in cadmium-treated rats. *Biol Trace Elem Res* 2006; **110**: 151-162

10 **Kanter M**, Demir H, Karakaya C, Ozbek H. Gastroprotective activity of Nigella sativa L oil and its constituent, thymoquinone against acute alcohol-induced gastric mucosal injury in rats. *World J Gastroenterol* 2005; **11**: 6662-6666

11 **El Shenawy NS**, Soliman MF, Reyad SI. The effect of antioxidant properties of aqueous garlic extract and Nigella sativa as anti-schistosomiasis agents in mice. *Rev Inst Med Trop Sao Paulo* 2008; **50**: 29-36

12 **Uz E**, Bayrak O, Uz E, Kaya A, Bayrak R, Uz B, Turgut FH, Bavbek N, Kanbay M, Akcay A. Nigella sativa oil for prevention of chronic cyclosporine nephrotoxicity: an experimental model. *Am J Nephrol* 2008; **28**: 517-522

13 **Farrag AR**, Mahdy KA, Abdel Rahman GH, Osfor MM. Protective effect of Nigella sativa seeds against lead-induced hepatorenal damage in male rats. *Pak J Biol Sci* 2007; **10**: 2809-2816

14 **Kanter M**, Coskun O, Budancamanak M. Hepatoprotective effects of Nigella sativa L and Urtica dioica L on lipid peroxidation, antioxidant enzyme systems and liver enzymes in carbon tetrachloride-treated rats. *World J Gastroenterol* 2005; **11**: 6684-6688

15 **Gali-Muhtasib H**, Diab-Assaf M, Boltze C, Al-Hmaira J, Hartig R, Roessner A, Schneider-Stock R. Thymoquinone extracted from black seed triggers apoptotic cell death in human colorectal cancer cells via a p53-dependent mechanism. *Int J Oncol* 2004; **25**: 857-866


**S- Editor** Cheng JX **L- Editor** Wang XL **E- Editor** Zheng XM

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*World J Gastroenterol* 2009 May 7; 15(17): 2172
***World Journal of Gastroenterology*** ISSN 1007-9327


*ACKNOWLEDGMENTS*

# Acknowledgments to reviewers of *World Journal of Gastroenterology*

Many reviewers have contributed their expertise and time to the peer review, a critical process to ensure the quality of *World Journal of Gastroenterology*. The editors and authors of the articles submitted to the journal are grateful to the following reviewers for evaluating the articles (including those published in this issue and those rejected for this issue) during the last editing time period.

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*World J Gastroenterol* 2009 May 7; 15(17): 2173
***World Journal of Gastroenterology*** ISSN 1007-9327


# 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
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*World J Gastroenterol* 2009 May 7; 15(17): 2174-2176
***World Journal of Gastroenterology*** ISSN 1007-9327


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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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