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EDITORIAL

Non-viral causes of hepatocellular carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is the most common primary liver malignancy and represents an international public health concern as one of the most deadly cancers worldwide. The main etiology of HCC is chronic infection with hepatitis B and hepatitis C viruses. However, there are other important factors that contribute to the international burden of HCC. Among these are obesity, diabetes, non-alcoholic steatohepatitis and dietary exposures. Emerging evidence suggests that the etiology of many cases of HCC is in fact multifactorial, encompassing infectious etiologies, comorbid conditions and environmental exposures. Clarification of relevant non-viral causes of HCC will aid in preventative efforts to curb the rising incidence of this disease.

Key words: Hepatocellular carcinoma; Etiology; Non-alcoholic steatohepatitis; Obesity; Diabetes; Alcohol; Tobacco; Oral contraceptive

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INTRODUCTION

Hepatocellular carcinoma (HCC) represents an international public health concern as one of the most common and deadly cancers worldwide. Globally, HCC accounts for 85%-90% of primary liver cancers[1] and its lethality is underscored by the fact that it is the third most frequent cause of cancer-related mortality[2]. In those patients who are not transplant candidates, HCC is particularly lethal, with a 5-year survival of less than 5%[3]. In the United States, the incidence of HCC appears to be increasing, with a more than twofold increase observed from 1976 to 2002 (Figure 1)[1,3,4]. A significant proportion of this increase is accounted for by the growing prevalence of hepatitis C virus (HCV) infection[5]. However, other potential causes of HCC are garnering close attention.

Increased body mass index and diabetes with subsequent development of non-alcoholic steatohepatitis (NASH) represent significant risk factors for HCC. This is especially concerning in light of the growing epidemic of obesity in adults and children over the past 25 years[1,5-8]. Other non-viral causes of HCC include iron overload syndromes, alcohol use, tobacco use, oral contraceptive use, aflatoxin exposure and betel quid chewing, a prevalent habit in the developing world. Emerging evidence suggests that the etiology of many cases of HCC is in fact multifactorial, including both viral infections and non-viral environmental and dietary exposures. This review focuses on the non-viral causes of HCC.

HEREDITARY HEMOCHROMATOSIS AND IRON OVERLOAD SYNDROMES

Hereditary hemochromatosis, a condition characterized by excess iron absorption, is caused by mutations in the

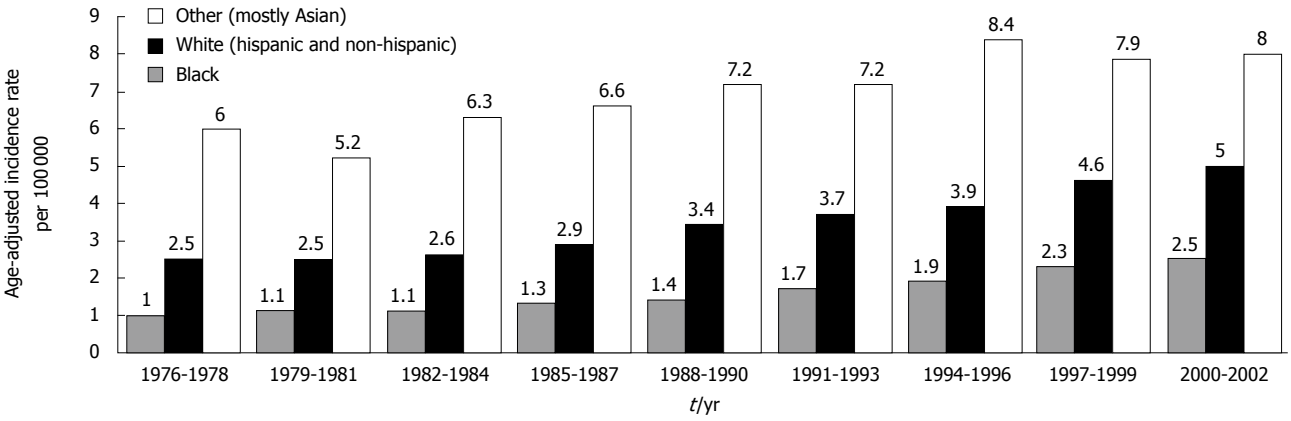

Figure 1 Average yearly, age-adjusted incidence rates for hepatocellular carcinoma men and women in the United States shown for 3-year intervals between 1976 and 2002. Whites included approximately 25% Hispanics, whereas other race was predominantly (88%) Asian. Source: Surveillance, Epidemiology, and End Results (SEER) Program (www.seer.cancer.gov) SEER*Stat Database: Incidence-SEER 13 Regs Public-Use, Nov 2004 Sub (1973-2002 varying), National Cancer Institute, DCCPS, Surveillance Research Program, Cancer Statistics Branch, released April 2005, based on the November 2004 submission. Reprinted from El-Serag HB, Rudolph KL. Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. *Gastroenterology* 2007; 132: 2557-2576, Copyright (2007), with permission from Elsevier[1].

HFE gene and/or other mutations in the iron metabolism machinery. This condition represents one of the most common autosomal recessive genetic disorders, affecting as many as 1 in 200 people of Northern European descent[9-11]. The *HFE* gene is required for efficient *in vivo* iron metabolism and two mutations within the *HFE* gene product, C282Y and H63D, have been well described in patients with hereditary hemochromatosis[10]. The C282Y mutation, which results in a base pair substitution in which tyrosine is substituted for cysteine at amino acid 282, is found in the homozygous state in up to 83% of patients with hereditary hemochromatosis[10]. The H63D mutation, characterized by substitution of histidine with aspartic acid at codon 63, is present in a minority of cases of hereditary hemochromatosis either in a homozygous state or with one copy of the C282Y mutation, a state referred to as a compound heterozygote[10]. The clinical significance of this latter mutation within the *HFE* gene, however, continues to be controversial.

The altered iron metabolism seen in hereditary hemochromatosis leads to excess iron storage in the liver and the subsequent development of liver dysfunction. Although other organs systems are also susceptible to iron overload, the liver bears the majority of malignant disease, with those patients with hereditary hemochromatosis being 20 times more likely to develop liver cancer than all other cancers combined[12].

Several population-based and case-control studies have shown that the diagnosis of hereditary hemochromatosis confers a consistent and markedly elevated risk for the development of HCC[12-17]. A sentinel study from the US National Center for Health Statistics found that patients who were diagnosed with hereditary hemochromatosis and who died were 23-fold more likely to have liver cancer compared to those without a diagnosis of hemochromatosis [Proportionate Mortality Ratio (PMR) 22.5, 95% CI: 20.6-24.6)[13]. In addition, the relationship between hereditary hemochromatosis and HCC is modified by diabetes, sex and genetics. Subjects with liver

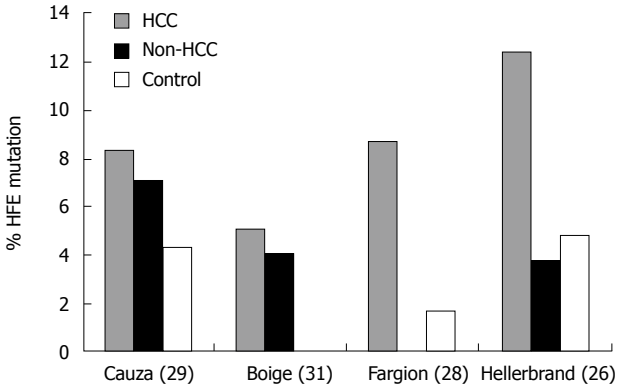

Figure 2 Prevalence of HFE mutations among patients with hepatocellular carcinoma, cirrhosis without hepatocellular carcinoma (non-hepatocellular carcinoma), and normal controls. Reprinted from Kowdley KV. Iron, hemochromatosis, and hepatocellular carcinoma. *Gastroenterology* 2004; 127: S79-S86, Copyright (2004), with permission from Elsevier [24]. HCC: Hepatocellular carcinoma.

cancer and concomitant diabetes mellitus were 82 times more likely to have a diagnosis of hemochromatosis[13]. Furthermore, a population-based study from Scandinavia found that men with hemochromatosis had a 29-fold increase in risk of liver cancer, whereas women with hemochromatosis had a sevenfold increase in risk[12]. Lastly, highlighting the genetic predisposition of disease and its consequences, an analysis of 5973 first degree relatives of patients with hemochromatosis found that these subjects had a nearly twofold increase in risk of HCC[12].

The presence of a single copy of the C282Y HFE gene mutation, although not diagnostic for hereditary hemochromatosis, has been studied to determine its prevalence and clinical significance in patients with HCC (Figure 2)[18-23]. Researchers comparing 81 patients with cirrhosis and HCC to 128 normal controls observed a significantly higher prevalence of the C282Y mutation in patients with HCC[18]. Another group observed that patients with HCC had a higher frequency of the C282Y mutation when compared to cirrhotic controls without HCC and healthy controls[19].

Additionally, they demonstrated that those subjects with the C282Y mutation had higher levels of serum ferritin, transferrin saturation, and hepatic iron deposition when compared to those without the C282Y mutation[19]. These studies suggest that increased iron load in HCC patients with a C282Y mutation exerts a cause and effect relationship in hepatocarcinogenesis[18,19]. This risk of HCC in patients with the C282Y mutation may not be equally conferred to all however. A recent prospective cohort study from France found that the C282Y mutation and iron overload were associated with a significantly increased risk of HCC among patients with alcoholic cirrhosis but not among those with HCV-related cirrhosis[23].

Contrary to the data presented above, two well-executed European studies did not find a significant difference in the prevalence of the C282Y mutation between patients with and without HCC[21,22]. Researchers from France observed comparable proportions of the C282Y heterozygous state in 133 cirrhotic patients with HCC and 100 without[21]. Likewise, in another cohort of 162 consecutive patients with HCC, the majority with cirrhosis, the frequency of the C282Y mutation did not differ from historical healthy controls or patients with HCV[22]. Concrete conclusions from these studies might be elusive, however, because of the small sample sizes, differences in the prevalence of the C282Y mutation in the respective populations, and referral bias to tertiary care centers[24].

More studies are therefore needed to determine correctly, in larger populations, the prevalence and effect of a single copy of the C282Y mutation. Additionally, on an individual basis, further study is needed to better characterize the comorbid, demographic and genetic factors that play a role in the risk of HCC in those with a single copy of the C282Y mutation.

It has also been proposed that the H63D mutation is not directly associated with hemochromatosis[10,25]. Certainly, none of the aforementioned studies observed a significant difference in the prevalence of the H63D mutation between patients with and without HCC[18-22]. Future studies are needed to assess further the relationship between this *HFE* gene mutation and the development of HCC.

Hereditary hemochromatosis is only one of the iron overload syndromes that leads to excessive iron deposition in the liver and other tissues. In fact, those patients with excess total body iron secondary to other etiologies have been shown to have a higher risk of HCC in the absence of genetic hemochromatosis[26-28]. Studies have suggested that conditions such as β thalassemia or iron overload in people of African descent might be associated with an increased risk of HCC[27,29,30]. One such study found that African iron loaded subjects had a 10-fold increase in the risk of developing HCC after adjusting for viral hepatitis, alcohol use and environmental exposures, such as aflatoxin[27]. Regardless of etiology, iron overload is not a benign condition and when recognized, surveillance for HCC should be undertaken.

NON-ALCOHOLIC FATTY LIVER DISEASE

Several case reports and subsequent observational studies have proposed that non-alcoholic fatty liver disease (NALFD), and more specifically, NASH, confers an elevated risk of developing HCC (Figure 3)[31]. NAFLD is a spectrum of clinical disease that ranges from benign or bland steatosis to NASH. The latter stage of this disease, through a process of chronic inflammation and subsequent hepatic fibrosis, can lead to cirrhosis[32]. The presence of cirrhosis itself is an independent risk factor for the development of HCC[33]. To characterize the natural history of NALFD, 420 patients identified in Olmstead County, MN, USA with the disorder were followed for an average of 7 years to determine overall mortality as well as liver related morbidity and mortality. In this population-based study, NAFLD was associated with a 34% increase in mortality and a significant increase in the risk of HCC, with two cases or 0.5% being diagnosed over the period of follow-up[34]. In subjects with NASH-related cirrhosis, however, the rate of HCC approached 10%[34]. These findings are well aligned with a series of studies from Japan. In one report, among 82 NASH patients treated from 1990 through 2001, six patients with HCC were identified over 11 years of follow-up[35]. All six patients developed HCC

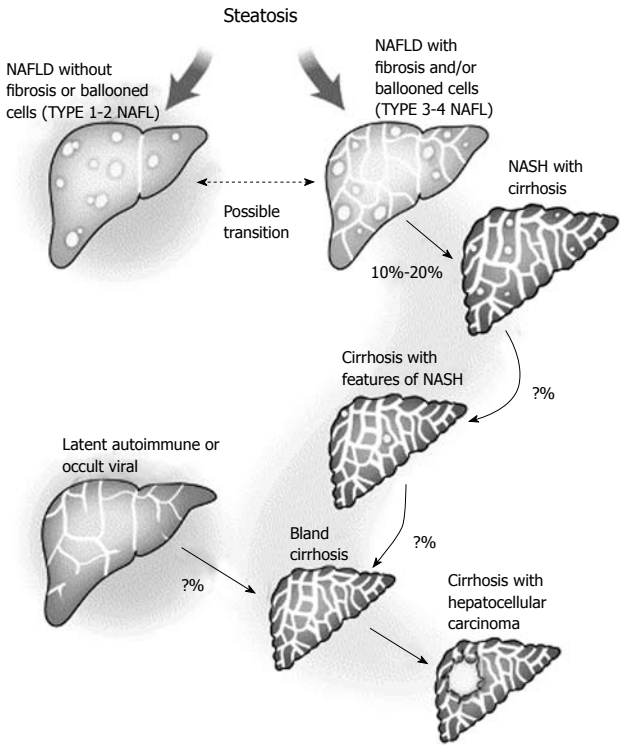

Figure 3 Progression of non-alcoholic fatty liver disease to cryptogenic cirrhosis. The explanation for the disappearance of steatosis remains uncertain but it is likely to be multifactorial and to involve changes in blood flow and exposure to fat-promoting hormones, as well as possible changes in the intracellular metabolism as a result of long-standing exposure to lipid peroxidation. Theoretically, this could represent a form of lipoatrophy that occurs within the fat-storing hepatocytes. Other forms of chronic liver disease may also present with a well-established bland cirrhosis. Efforts are needed to define better residual markers of past silent disease to improve our understanding of cryptogenic cirrhosis. Reprinted from Caldwell SH, Crespo DM. The spectrum expanded: cryptogenic cirrhosis and the natural history of non-alcoholic fatty liver disease. *J Hepatol* 2004; 40: 578-584, Copyright (2004), with permission from Elsevier[31]. NALFD: Non-alcoholic fatty liver disease; NASH: Non-alcoholic steatohepatitis.

Table 1 Characteristics of cohort studies included in the meta-analysis

Study	Country	No. of cases (men/women)	Study participants	Assessment of exposure	Adjustments
Møller *et al* (1994)	Denmark	22/36	Men: 14531 Women: 29434	Discharge diagnosis of obesity	Age
Wolk *et al* (2001)	Sweden	15/13	Men: 8165 Women: 19964	Discharge diagnosis of obesity	Age, calendar year
Nair *et al* (2002)	USA	659[1]	Men and women: 19271[1]	Measured	Age, sex, race, diabetes
Calle *et al* (2003)	USA	620/345	Men: 404576 Women: 495477	Self-reported	Age, race, education, marital status, smoking, physical activity, aspirin use, estrogen-replacement therapy (women), alcohol, dietary factors
Samanic *et al* (2004)	USA	322 whites/38 blacks	White men: 3668486 Black men: 832214	Discharge diagnosis of obesity	Age, calendar year
Kuriyama *et al* (2005)	Japan	69/31	Men: 12485 Women: 15054	Self-reported	Age, type of health insurance, smoking, intakes of alcohol, meat, fish, fruits, vegetables, bean-paste soup[2]
Batty *et al* (2005)	UK	51	Men: 18403	Measured	Age, employment grade, marital status, physical activity, smoking, other[3]
Oh *et al* (2005)	Korea	3347	Men: 781283	Measured	Age, area of residence, family history of cancer, smoking, exercise, alcohol
Rapp *et al* (2005)	Austria	57	Men: 67447	Measured	Age, occupational group, smoking
N'Kontchou *et al* (2006)	France	220[1]	Men and women: 771[1]	Measured	Age, sex, cirrhosis cause, diabetes
Samanic *et al* (2006)	Sweden	297	Men: 362552	Measured	Age, smoking

[1]Patients with cirrhosis; [2]ORs for women were further adjusted for age at menarche, age at end of first pregnancy, and menopausal status; [3]Other factors adjusted for include disease at entry, weight loss in the last year, height-adjusted FEV1, triceps skinfold thickness, blood pressure-lowering medication, blood pressure, plasma cholesterol, glucose intolerance, and diabetes. Reprinted by permission from Macmillan Publishers Ltd: [Br J Cancer]. Larsson SC, Wolk A. Overweight, obesity and risk of liver cancer: a meta-analysis of cohort studies. *Br J Cancer* 2007; 97: 1005-1008, copyright (2007)[42].

in the setting of NASH-related cirrhosis[35]. In an update to this original observation, over a 17-year period, the authors found that among 382 patients with NASH, HCC was diagnosed in 34, 9% of the cohort, with 11 patients diagnosed during a 40-mo mean follow-up[36]. Comparing those NASH patients with and without HCC, multivariate logistic regression analysis identified older age (OR: 1.1, 95% CI: 1.03-1.2) and advanced hepatic fibrosis (OR: 4.2, 95% CI: 1.8-9.7) as independent predictors for the development of HCC[36]. In a prospective study of 118 patients with NASH and advanced liver fibrosis from the same cohort, the observed 5-year cumulative incidence of HCC was 7.6%, with HCC accounting for 46% of all fatalities[36]. In summary, these data highlight an association between NASH cirrhosis and an increase in the incidence of HCC over that of the general population. Therefore, regular HCC surveillance is imperative in patients with NASH cirrhosis.

The impact of NASH on the incidence of HCC may well be underestimated. In advanced fibrosis, an absence of steatosis may be appreciated, a finding which can obscure identification of the underlying etiology of liver injury in these patients. In this case, patients might be classified as having cryptogenic cirrhosis. In a United States study that examined 105 consecutive patients with HCC, after HCV, cryptogenic cirrhosis was the most common etiology of liver injury[37]. Among patients presenting with cryptogenic cirrhosis, 58% had a body mass index (BMI) $\geqslant$ 30, 47% had diabetes, and 50% had a prior histological diagnosis of NASH or clinical characteristics consistent with NAFLD. Furthermore, only 23% of subjects with cryptogenic cirrhosis were undergoing surveillance for HCC in comparison to 61% of subjects who had a history of HCV-related liver disease[37]. Clearly, these observations emphasize the importance of HCC surveillance in this group of patients and the failure thus far to appropriately screen for HCC in this disease process.

OBESITY

The prevalence of obesity has increased to epidemic proportions over the last three decades. Excess body mass is classified as overweight if the BMI is > 25 kg/m^2 and < 30 kg/m^2, or obese if the BMI is $\geqslant$ 30 kg/m^2. In addition to the increase in an array of disease processes observed with being overweight or obese, both classifications of excess body mass are associated with a higher risk of developing all cancers, including liver cancer[38]. In one population-based study from Sweden, 28 cases of HCC were diagnosed in 28129 patients from 1965 to 1993, thus conferring an almost threefold higher risk of HCC in obese patients[39]. A recent European case-control study observed a significantly increased risk of HCC among obese (OR: 3.5, 95% CI: 1.3-9.2) or diabetic (OR: 3.5, 95% CI: 1.6-7.7) patients without viral hepatitis. This risk of HCC was even greater if both obesity and diabetes were present comorbid conditions (OR: 11.8, 95% CI: 2.7-51.9)[40]. A Danish study further confirmed these results, finding a twofold increase in liver cancer incidence in obese subjects compared to non-obese subjects[41].

A meta-analysis of 11 cohort studies that evaluated the association between being overweight or obese and liver

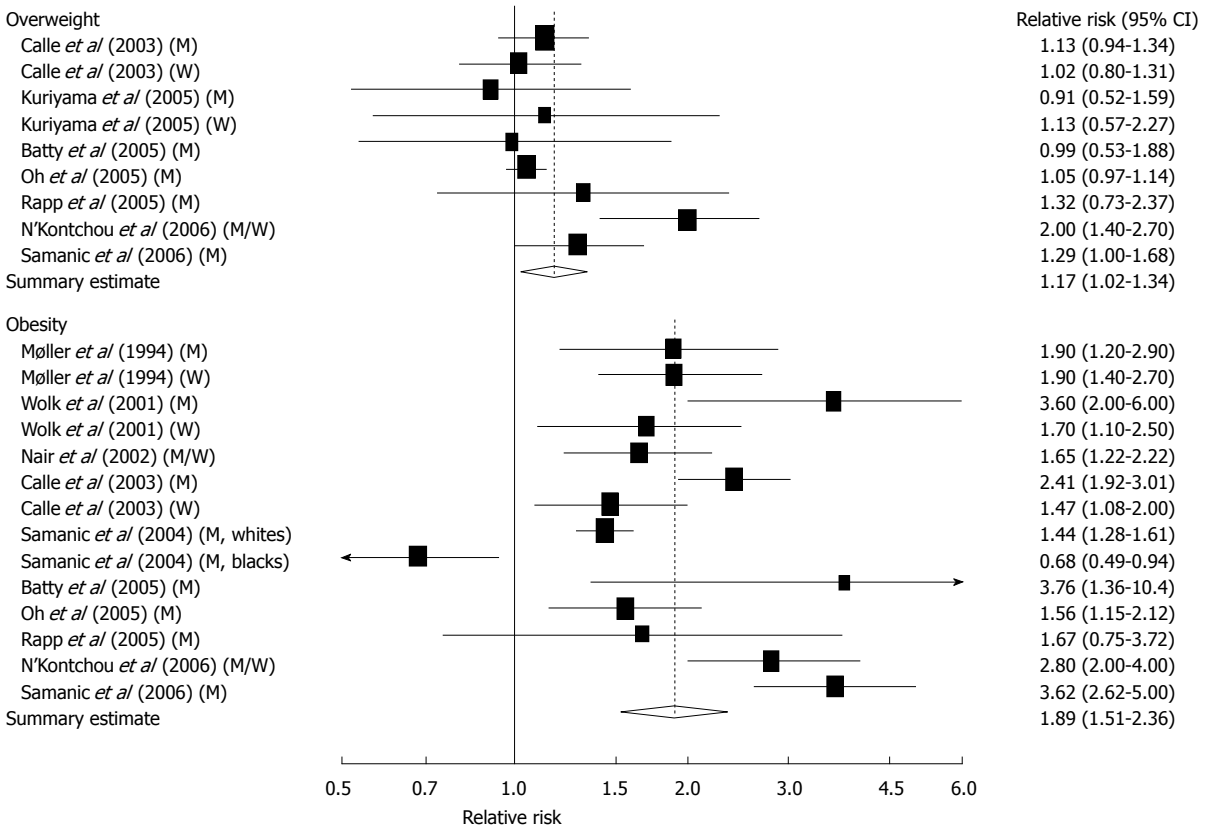

Figure 4 Relative risks of liver cancer associated with overweight and obesity. Relative risk estimates are for overweight and obese persons compared with normal weight persons. Tests for heterogeneity: overweight, Q = 16.83, P = 0.03, I^2 = 52.5%; obesity, Q = 88.03, P < 0.001, I^2 = 86.4%. M: Men; W: Women[42]. Reprinted by permission from Macmillan Publishers Ltd: [Br J Cancer]. Larsson SC, Wolk A. Overweight, obesity and risk of liver cancer: a meta-analysis of cohort studies. *Br J Cancer* 2007; 97: 1005-1008, copyright (2007)[42].

cancer was published in 2007, and clarified the risk of development of HCC (Table 1)[42]. Of the included studies, seven examined a total of 5037 overweight patients and 10 examined 6042 obese patients[42]. Patients who were overweight had a 17% increase in risk of developing HCC, whereas obese patients had an 89% increase in risk (Figure 4)[42]. Based on the prevalence of HCC, it was estimated that 28% of HCC cases in men and 27% in women were due to being overweight or obese[42].

In addition to an increased risk of developing HCC, overweight or obese patients appear to be at increased risk for HCC-related mortality. In a population-based study of cancer mortality and BMI, men with a BMI of 30-34.9 were found to have a twofold increase in the risk of death from HCC, with a 4.5-fold increase noted in men with BMI > 35[38].

Lastly, *via* the pathway of the metabolic syndrome with resultant NASH cirrhosis, obese patients have been found to be at an increased risk for HCC occurrence. Many lines of evidence point to the role of cirrhosis as a mediator in these patients. Firstly, patients presenting with cryptogenic cirrhosis were found to have a significantly higher prevalence of obesity than patients with cirrhosis from non-alcoholic hepatitis C or autoimmune liver disease, but a similar prevalence of obesity when compared to patients with documented NASH[43]. These data are supported by a case-control study in which 49 patients with cryptogenic cirrhosis were compared to 98 matched controls with an established cause of cirrhosis. In that study, obesity was significantly more prevalent in the cryptogenic cirrhosis patients[44]. Additionally, a retrospective analysis of 19 271 American patients who had undergone liver transplantation found that there were 653 cases of HCC, and those with a diagnosis of cryptogenic cirrhosis had an 11-fold increase in the risk of having HCC[45]. Therefore, being overweight and obesity, secondary to cryptogenic cirrhosis, or more likely undiagnosed NASH cirrhosis, can increase the risk of developing HCC. Clearly, these data suggest that screening is important for diagnosis of asymptomatic HCC and highlight the need for surveillance in this population.

DIABETES

Diabetes has been found to increase the risk of developing chronic liver disease and HCC[46]. Studies that have compared patients with cryptogenic cirrhosis to patients with a known etiology of their cirrhosis have shown a significantly higher prevalence of diabetes among the latter group[43,44]. Again, as noted with the overweight and obese, a similar prevalence of diabetes has been observed among patients with cryptogenic and NASH cirrhosis[43].

In addition to increasing the prevalence of chronic liver disease, diabetes has also been shown to be an independent risk factor for the development of HCC. In a recent systematic review of 13 case control studies, 11

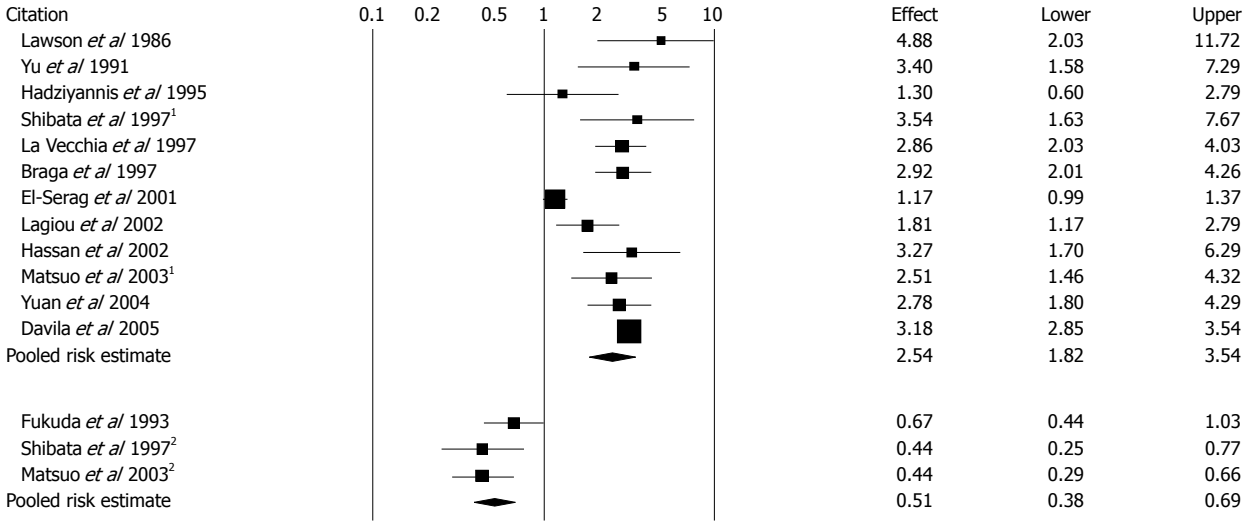

Figure 5 Unadjusted ORs and 95% CIs for the association between diabetes and hepatocellular carcinoma in 13 case-control studies. [1]Population-based control groups; [2]Hospital-based control groups. Black diamonds indicate weighted average of all studies; Black boxes indicate point estimates for ORs[47] Reprinted with El-Serag HB, Hampel H, Javadi F. The association between diabetes and hepatocellular carcinoma: a systematic review of epidemiologic evidence. *Clin Gastroenterol Hepatol* 2006; 4: 369-380, Copyright (2006), with permission from Elsevier[47].

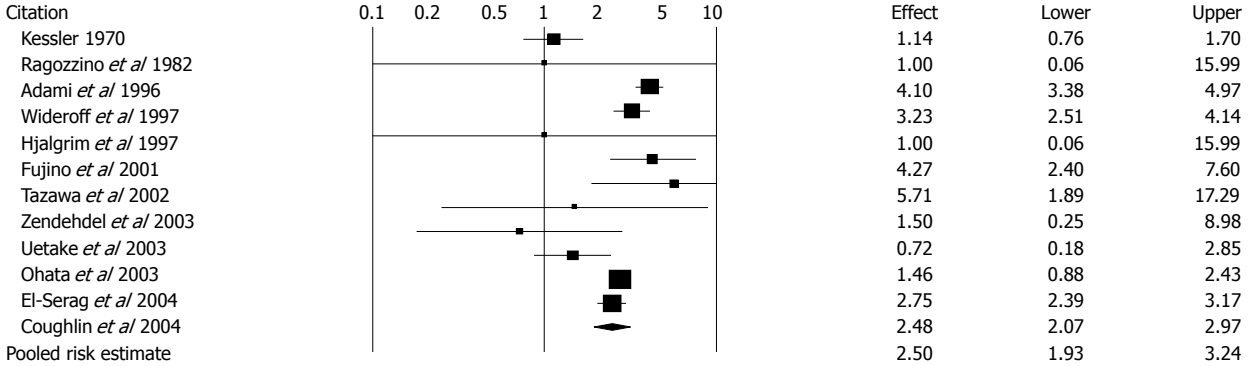

Figure 6 Unadjusted risk ratios and 95% CIs for the association between diabetes and hepatocellular carcinoma in 12 cohort studies. Black diamond indicates weighted average of all studies; Black boxes indicate the point estimates for risk ratios[47]. Reprinted from El-Serag HB, Hampel H, Javadi F. The association between diabetes and hepatocellular carcinoma: a systematic review of epidemiologic evidence. *Clin Gastroenterol Hepatol* 2006; 4: 369-380, Copyright (2006), with permission from Elsevier[47].

supported an association between diabetes and the development of HCC[47]. Among the 13 case-control studies, subjects with diabetes were found to have a twofold increase in the risk of HCC; an association that was further strengthened by excluding studies with significant heterogeneity (Figure 5)[47]. This association was also appreciated amongst 12 cohort studies evaluated (Figure 6). The presence of diabetes remained an independent risk factor for HCC after adjustment for alcohol use or viral hepatitis in the studies that evaluated these factors[47]. However, as dictated by the limitations of the studies available in the literature, further well-defined studies are required to account for dietary factors and obesity.

DIET

Several studies have examined whether alterations in diet have an effect on the risk of HCC. A trial from Italy has examined a broad range of dietary habits among 185 patients with HCC and 412 patients without cancer[48,49]. Those with HCC were more likely to consume a large amount of calories, were five times more likely to be former drinkers, and were 30 times more likely to be infected with either HCV or hepatitis B virus (HBV). Among dietary compounds, consumption of iron and thiamine were associated with a significant threefold and twofold increase in risk of HCC, respectively. Conversely, β-carotene and linoleic acid consumption was associated with a reduced risk of HCC[48]. An association between intake of iron was also evaluated according to the presence or absence of viral hepatitis[48]. When compared to appropriate controls, consumption of iron among patients without viral hepatitis was associated with a significantly increased risk of HCC[48]. This increase in risk was not conferred to those with HCV or HBV. In a similar study, those subjects with consumption in the highest quartile for yogurt and milk, white meat and eggs had a significantly lower likelihood of developing HCC[50]. This effect was observed in patients with and without viral hepatitis[50].

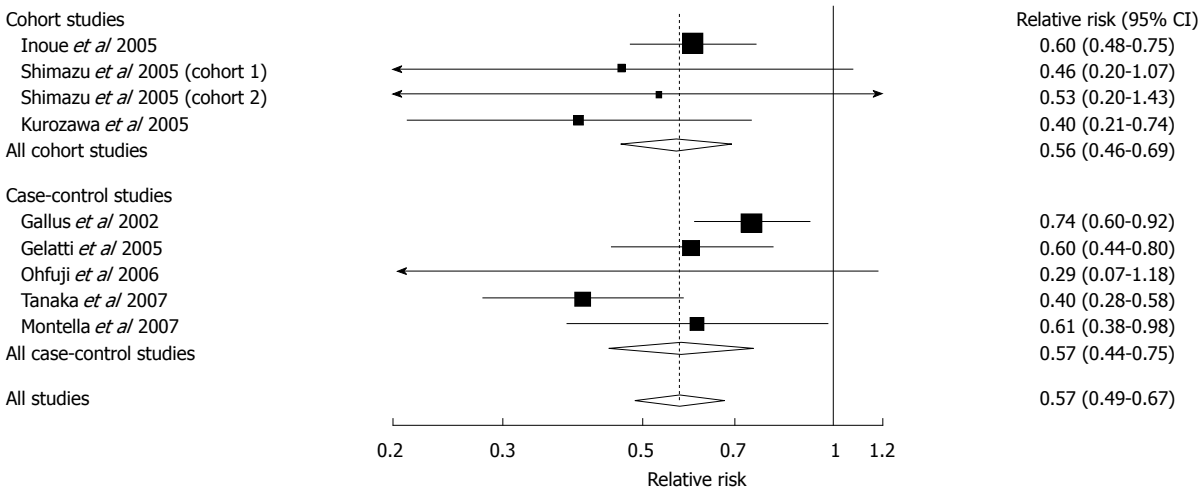

Figure 7 Relative risks of liver cancer associated with coffee consumption (per 2 cups/d increment). Squares represent study-specific relative risk estimates (size of the square reflects the study-specific statistical weight, that is, the inverse of the variance); horizontal lines represent 95% CIs; diamonds represent summary relative risk estimates with corresponding 95% CIs. Tests for heterogeneity: all studies, Q = 11.56, P = 0.17, I^2 = 30.8%; cohort studies, Q = 1.74, P = 0.63, I^2 = 0%; case-control studies, Q = 9.28, P = 0.05, I^2 = 36.9%. Reprinted from Larsson SC, Wolk A. Coffee consumption and risk of liver cancer: a meta-analysis. *Gastroenterology* 2007; 132: 1740-1745, Copyright (2007), with permission from Elsevier[66].

Other studies from Japan and Europe have found those who consume a large amount of green vegetables have a significantly lower likelihood of developing HCC[51-53]. One study has shown that eating green vegetables daily, as compared with consumption fewer times per week, had a protective effect against the development of HCC (OR: 0.75, 95% CI: 0.60-0.95)[51]. On the contrary, a Greek study has found no association between vegetable intake and reduction in the risk of developing HCC[54].

In summary, there is evidence to suggest that consumption of yogurt and milk as well as vitamin supplements offers a protective effect against HCC. The enthusiasm for these findings however should be tempered by the fact that the majority of these studies were retrospective in nature.

COFFEE

In addition to its reported association with reductions in bladder cancer and colorectal cancer, coffee consumption has also been extensively studied and appears to have a potentially favorable effect on the prevention of liver diseases, including HCC[55,56]. There are several hypotheses that could explain why consuming coffee attenuates the risk of developing HCC. One hypothesis argues that coffee intake lowers serum levels of γ-glutamyl transferase (GGT), which is associated with a lower incidence of HCC[56-59]. Coffee consumption has also been linked to a lower incidence of cirrhosis, which is a major risk factor for the development of HCC[56].

An analysis of two large prospective studies of > 70 000 participants in Japan has shown that those who drank one or more cups coffee daily had a significantly lower risk of developing HCC[60]. A case-control study of 2746 people has found that those who drank three or more cups of coffee were 40% less likely to develop HCC[56]. Similar results have also been found in an array of studies conducted in Europe and Japan[60-65].

Additionally, two meta-analyses that have examined the association between coffee drinking and the risk of developing HCC have recently been published. The first was inclusive of four cohort studies and five case-control studies[66]. In the pooled analysis, a 43% lower risk of developing HCC was found for those who drank more than two cups of coffee per day (RR: 0.57, 95% CI: 0.49-0.67) (Figure 7)[66]. The second meta-analysis examined four cohort studies from Japan and six from Japan and Southern Europe[67]. There were differing definitions of low and high coffee consumption, however, the results of the studies were consistent. In summary, those who drank any coffee compared to non-drinkers had a significantly lower risk of HCC (RR: 0.59, 95% CI: 0.49-0.72). The greater the coffee consumption, the greater the attenuation in HCC risk. Low coffee consumption was associated with a 30% reduction in risk and high consumption with a 55% reduction in HCC risk (Figure 8)[67].

Although these results are impressive and consistent, one must consider that the findings of an inverse relationship between coffee consumption and the risk of HCC might be influenced by bias. Coffee metabolism is impaired in cirrhotic livers as compared to the normal liver. This altered metabolism generates an increase in the untoward side effects of the beverage. Therefore, the presence of liver disease might lead affected patients to consume less coffee. This could result in a falsely negative association. Therefore, the potential bias of this association in the liver disease patient cannot be discounted.

ALCOHOL

The mechanism by which alcohol consumption increases the risk of HCC is primarily through the development of cirrhosis. It has been suggested that heavy alcohol consumption of > 80 g/d ethanol for at least 5 years increases the risk of HCC by nearly fivefold[68]. The risk appears to be proportional to the amount of alcohol consumed.

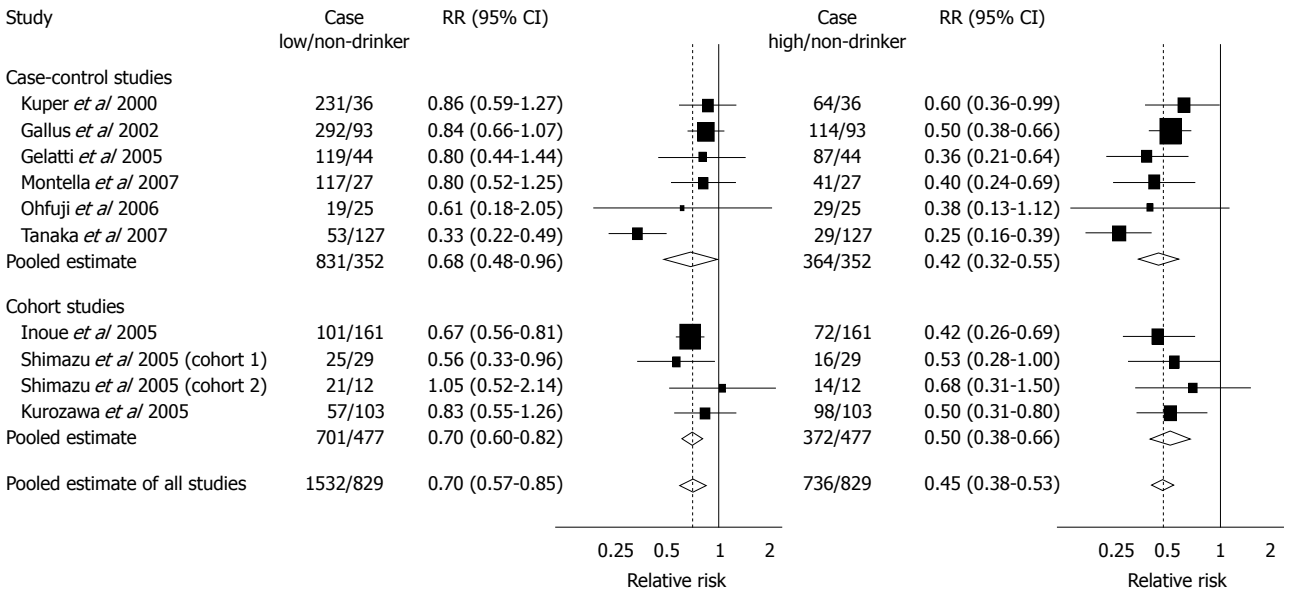

Figure 8 Summary RRs of hepatocellular carcinoma for low or moderate and high coffee drinkers *vs* non-drinkers from case-control and cohort studies. Low or moderate consumption was defined as < 3 cups per day for Gallus *et al*, Gelatti *et al*, Inoue *et al* and Montella *et al* and as < 1 cup per day for Ohfuji *et al*, Tanaka *et al*, Kurozawa *et al*, and Shimazu *et al*; High consumption was defined as ⩾ 3 cups per day for Gallus *et al*, Gelatti *et al*, Inoue *et al*, and Montella *et al* and as ⩾ 1 cup per day for Ohfuji *et al*, Tanaka *et al*, Kurozawa *et al*, and Shimazu *et al*. Bravi F, Bosetti C, Tavani A, Bagnardi V, Gallus S, Negri E, Franceschi S, La Vecchia C. Coffee drinking and hepatocellular carcinoma risk: a meta-analysis. *Hepatology* 2007; 46: 430-435[67]. Copyright 2007, John Wiley & Sons, Inc. Reproduced with permission of John Wiley & Sons, Inc.

As characterized by a meta-analysis, relative risks of 1.19 (95% CI: 1.12-1.27), 1.40 (95% CI: 1.25-1.56), and 1.81 (95% CI: 1.50-2.19) were associated with the consumption of 25, 50 and 100 g/d alcohol, respectively[69].

In addition to a daily dose response, persistent alcohol consumption appears to have a long-term effect on the risk of HCC occurrence. A prospective case-control study from Japan has observed that heavy alcohol drinkers, defined as > 600 L of alcohol during a lifetime, had a fivefold increase in the risk of HCC in comparison to non-drinkers or those who consumed < 600 L of alcohol (OR: 5.19, 95% CI: 2.53-10.64)[70]. However, the risk of HCC among those who consume low or moderate levels of alcohol remains unknown[1].

An association between genetic polymorphisms of the enzymes participating in the metabolic pathway of ethanol and the increased risk of HCC in heavy alcohol drinkers has been also proposed as a mechanism by which HCC develops. The frequency of aldehyde dehydrogenase 2 (*ALDH2*) genotype polymorphisms is significantly associated with increased risk of HCC in heavy alcohol drinkers (OR: 2.53, 95% CI: 1.63-58.60)[70]. A study from Italy has observed that, among subjects who consumed > 100 g/d of ethanol and were bearers of the gluthatione S-transferase M1 (GSTM1) null genotype had twice the risk of HCC compared with bearers of the GSTM1 non-null genotype (OR: 8.5, 95% CI: 3.9-18.6 *vs* OR: 4.5, 95% CI: 2.0-10.0)[71].

SMOKING

Several studies have evaluated the association between smoking and development of primary liver cancer. A prospective cohort study including 4050 men aged ⩾ 40 years who were followed-up for an average length of 9 years observed that those who smoked had a threefold increased risk of primary liver cancer when compared to never smokers (RR: 3.3, 95% CI: 1.2-9.5)[72]. Additionally, a study from Korea has found a 50% increase in the risk of primary liver cancer for current male smokers compared to never smokers[73]. In contrast however, a recent population-based case-control study from the United States did not observe a significantly increased risk of primary liver cancer among current male smokers[74]. Male ex-smokers, however, had a significant increase in risk of primary liver cancer, which suggests that there is perhaps a dose or duration response underlying this association[72-74]. Such responses were further explored in the Korean Cancer Prevention study that included 1 283 112 subjects[75]. Although the amount of smoking did not alter the risk of HCC, the duration of smoking significantly increased the risk of HCC for subjects who had smoked for > 20 years when compared to those who had smoked for < 10 years[75].

The association between tobacco and liver cancer and its reliance on host factors such as genetics, sex, and an underlying history of viral hepatitis has also been explored. With respect to the role of genetics, a small study from Japan has evaluated 78 patients with HCC and genetic polymorphisms of tobacco and alcohol-related metabolizing enzymes and 138 hospital controls without cancer. They have demonstrated that cigarette smokers did not have a significantly increased risk of HCC when compared with non-smokers[70]. To analyze the effect of sex, a prospective cohort study that included 83 885 patients followed up for 8 years observed a positive association between smoking and HCC in women who smoked > 10 cigarettes per day (RR: 4.2, 95% CI: 1.3-13.8)[76]. However, no significant increase in the risk of HCC was

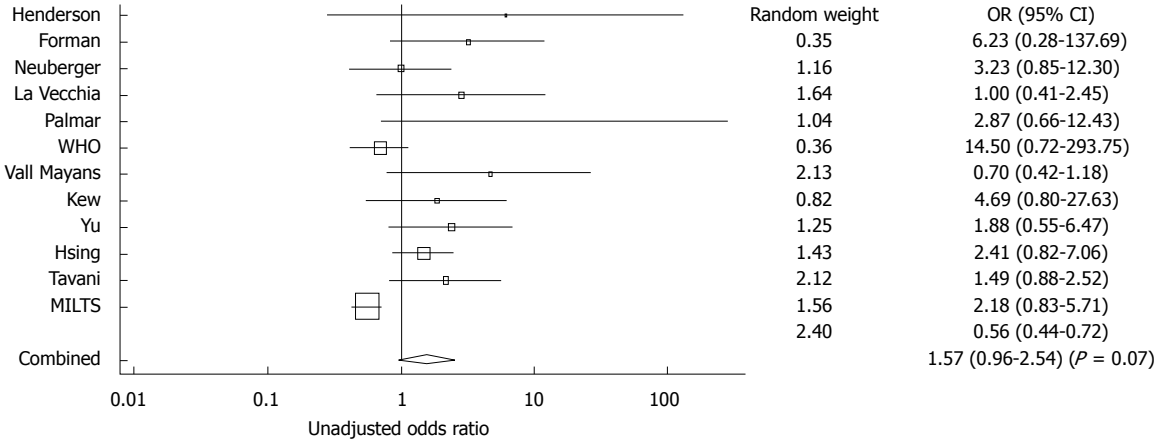

Figure 9 Forest plot showing adjusted OR and 95% CI for the association between oral contraceptive and hepatocellular carcinoma for the eight studies that included adjusted ORs. The diamond symbol indicates the weighted random pooled OR of all studies included in the analysis. Reprinted from Maheshwari S, Sarraj A, Kramer J, El-Serag HB. Oral contraception and the risk of hepatocellular carcinoma. *J Hepatol* 2007; 47: 506-513, Copyright (2007), with permission from Elsevier[95].

demonstrated among male smokers[76]. Additionally, to determine the effect of viral hepatitis on the association between HCC and tobacco, a prospective study of 12 008 men observed that smoking significantly increased the risk of HCC only in anti-HCV-positive patients but not in those who were anti-HCV-negative when compared to anti-HCV-negative nonsmoking individuals[77].

In addition to an increase in the risk of developing HCC, it is also suggested in the literature that smoking increases the risk of death in HCC. In the Korean Cancer Prevention cohort study, men who were current smokers had an increased risk of death from HCC[75]. Women who were current smokers did not have the same elevation in risk of HCC-related death as that observed in men[75]. These findings were further replicated in the Japan Collaborative Cohort (JACC) Study that analyzed 65 528 subjects aged 40-79 years[78]. In this cohort, an increased risk of death due to HCC was shown among current and ex-smokers[78]. Further analyses from the JACC cohort demonstrated that cigarette smoking significantly increased the risk of death from HCC in individuals positive for anti-HCV antibody[79].

ORAL CONTRACEPTIVES

Prior to the widespread use of oral contraceptives (OCs), benign liver tumors in young women were rarely observed. In the current case report literature, however, therapy with oral contraceptives appears to be associated with the development of benign liver tumors such as hepatic hemangioma, hepatocellular adenoma or focal nodular hyperplasia[80,81]. Although not well researched, it has been proposed that OCs might also be associated with malignant liver tumors including HCC[82,83]. Rarely, malignant transformation can occur within the context of hepatic adenomas. It is unclear, however, whether the use of OCs influences the likelihood of developing adenoma and that these benign tumors transform.

Within the literature, there have been 14 cases of hepatic adenoma with focal malignant transformation to HCC in women taking OCs[83-93]. The mean age of these patients at the time of diagnosis of malignant transformation was 36 years (range: 23-57 years) and the mean duration of OC use was 11 years (range: 1 mo-20 years)[83-93]. Although difficult to obtain from the literature, the frequency of HCC among hepatic adenomas appears to vary from 5% to 18%[89,92-94].

To evaluate further the risk of HCC in the setting of OC use, several observational studies have been conducted. A recent meta-analysis of 12 case-control studies, including 739 cases and 5223 controls, which evaluated the risk of HCC among women using OCs indicated that there was no increase in risk of HCC with short-term use; defined as < 5 years of exposure[95]. An analysis of all studies in the aforementioned meta-analysis yielded a pooled unadjusted OR of 1.57 (95% CI: 0.96-2.54)[95]. An adjusted analysis, which accounted for variables such as age, race and parity, did not yield significant findings (Figure 9)[95]. On the contrary, six studies have observed a significantly increased risk of HCC among women taking OCs for > 5 years; an increase in risk of 2-20-fold[95]. However, given the variable periods of duration used in each of the studies, a pooled estimate of risk could not be generated[95]. Based on these results, further studies are required to evaluate the association between OCs and the risk of HCC and how such risk is modified by duration of OC use. Additionally, it should be noted that an association between new-generation OCs with lower doses of hormones and the risk of HCC has not yet been explored.

BETEL QUID

The chewing of betel quid is woven into the cultural fabric of up to 20% of the world population. Betel quid consists of the nut of the *Areca catechu* palm (areca nut), betel leaf or fruit from *Piper betle* and red slaked paste[96]. These ingredients have been shown to have genotoxic, mutagenic and tumorigenic properties[97-102]. A case-con-

trol study from Taiwan has shown that betel quid chewing was an independent risk factor for liver cirrhosis (OR: 3.56, 95% CI: 1.41-8.96)[103].

Recently, two prospective case-control studies from Asia also have observed a significant association between betel quid chewing and the incidence of HCC. One such study included 263 pairs of age- and sex-matched patients with HCC and healthy controls and observed that betel quid chewing was an independent risk factor for HCC, with a threefold risk noted (OR: 3.49, 95% CI: 1.74-6.96). The aggregate risk increased with increasing duration and/or quantity of consumption[96]. These data were further supported by a study from Taiwan, including 420 age- and sex-matched patients with HCC and liver cirrhosis, liver cirrhosis only and healthy controls. In this study, a nearly sixfold and nearly twofold increased risk of HCC was observed in patients with HCC compared with healthy controls and patients with liver cirrhosis, respectively[104]. Additionally, they found an additive interaction between betel quid chewing and chronic HBV and/or HCV infection.

AFLATOXIN

Aflatoxin B1 (AFB1) is the major metabolite of the molds *Aspergillus fumigatus* and *Aspergillus parasiticus*. These molds grow on a variety of food products that are stored in warm and damp conditions or are cultivated in countries with hot and humid climates[1,105]. AFB1 induces a single nucleotide substitution in codon 249 in the *p53* tumor suppressor gene, which results in the change of the amino acid arginine to serine[106,107]. This mutation is present in up to 50% of patients with HCC who are indigenous to geographic regions with high exposure to AFB1[108-111]. On the other hand, this mutation is absent in patients with HCC from regions with low exposure to AFB1[112,113]. Moreover, it has been recently demonstrated that AFB1-albumin adducts in patients with HCC correlate significantly with the presence of plasma DNA hypermethylation and mutations in the *p16* and *p53* tumor suppressor genes[114].

Several studies have evaluated an association between the risk of HCC and exposure to AFB1. A prospective case-control study from China which included 18 244 middle-aged men showed that individuals with the presence of urinary aflatoxin biomarkers had a significantly increased risk of HCC after adjusting for HBV surface antigen seropositivity and cigarette smoking[115]. These data were further supported by a community-based cohort study from Taiwan which found that elevated AFB1 exposure measured by detectable AFB1-albumin adducts was an independent risk factor for HCC after adjustment for important confounders (OR: 5.5, 95% CI: 1.2-24.5)[116].

It should be stressed that areas with high exposure to AFB1 are also characterized by a high prevalence of HBV infection. AFB1 is independent of the risk conferred by HBV, however concomitant exposure to both HBV and AFB1 markedly increases the risk of HCC. When compared to those without HBV infection and absence of urinary AFB1 markers, the risk of HCC was 60 times higher in patients with HBV infection and a concomitant elevation of urinary AFB1 markers (RR: 59.4, 95% CI: 16.6-212.0)[115]. Patients with HBV infection and normal urinary AFB1 markers had sevenfold increase in risk of HCC when compared to appropriate controls[115].

CONCLUSION

Multiple non-viral factors have been implicated in the development of HCC. Hemochromatosis and iron overload syndromes have consistently been shown to dramatically increase the rate of HCC. Additionally, factors such as obesity and diabetes, which operate *via* NASH cirrhosis or perhaps independently, have also been demonstrated to increase the risk of HCC. This phenomenon has closely mirrored the epidemic of obesity over the last 15-25 years.

With respect to other exposures, although alcohol and tobacco clearly increase the risk of HCC development and mortality, other exposures such as coffee and high levels of vegetable consumption may be protective against this condition. Further studies are urgently needed to determine the pathogenesis that underlies the occurrence of HCC in the setting of these exposures, as well as the way in which such risk is modified by environmental and host characteristics such as genetics.

Clarification of relevant non-viral causes of HCC will help to focus clinicians on those risk factors that are modifiable. With more information, future surveillance efforts will be more appropriately targeted toward populations at greatest risk. This multilevel preventative approach will hopefully lead to a reduction in incidence of non-viral HCC, and a decrease in the patient morbidity and mortality as well as the societal economic burden associated with HCC.

REFERENCES

1 **El-Serag HB**, Rudolph KL. Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. *Gastroenterology* 2007; **132**: 2557-2576

2 **Parkin DM**, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. *CA Cancer J Clin* 2005; **55**: 74-108

3 **El-Serag HB**, Mason AC. Rising incidence of hepatocellular carcinoma in the United States. *N Engl J Med* 1999; **340**: 745-750

4 **El-Serag HB**, Davila JA, Petersen NJ, McGlynn KA. The continuing increase in the incidence of hepatocellular carcinoma in the United States: an update. *Ann Intern Med* 2003; **139**: 817-823

5 **El-Serag HB**, Mason AC. Risk factors for the rising rates of primary liver cancer in the United States. *Arch Intern Med* 2000; **160**: 3227-3230

6 **James WP**. The epidemiology of obesity: the size of the problem. *J Intern Med* 2008; **263**: 336-352

7 **Regimbeau JM**, Colombat M, Mognol P, Durand F, Abdalla E, Degott C, Degos F, Farges O, Belghiti J. Obesity and diabetes as a risk factor for hepatocellular carcinoma. *Liver Transpl* 2004; **10**: S69-S73

8 **Schroeder SA**. Shattuck Lecture. We can do better--improving the health of the American people. *N Engl J Med* 2007; **357**: 1221-1228

9 **Edwards CQ**, Griffen LM, Goldgar D, Drummond C, Skolnick MH, Kushner JP. Prevalence of hemochromatosis among

11,065 presumably healthy blood donors. *N Engl J Med* 1988; **318**: 1355-1362
10 **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
11 **Powell LW**, Subramaniam VN, Yapp TR. Haemochromatosis in the new millennium. *J Hepatol* 2000; **32**: 48-62
12 **Elmberg M**, Hultcrantz R, Ekbom A, Brandt L, Olsson S, Olsson R, Lindgren S, Loof L, Stal P, Wallerstedt S, Almer S, Sandberg-Gertzen H, Askling J. Cancer risk in patients with hereditary hemochromatosis and in their first-degree relatives. *Gastroenterology* 2003; **125**: 1733-1741
13 **Yang Q**, McDonnell SM, Khoury MJ, Cono J, Parrish RG. Hemochromatosis-associated mortality in the United States from 1979 to 1992: an analysis of Multiple-Cause Mortality Data. *Ann Intern Med* 1998; **129**: 946-953
14 **Bradbear RA**, Bain C, Siskind V, Schofield FD, Webb S, Axelsen EM, Halliday JW, Bassett ML, Powell LW. Cohort study of internal malignancy in genetic hemochromatosis and other chronic nonalcoholic liver diseases. *J Natl Cancer Inst* 1985; **75**: 81-84
15 **Strohmeyer G**, Niederau C, Stremmel W. Survival and causes of death in hemochromatosis. Observations in 163 patients. *Ann N Y Acad Sci* 1988; **526**: 245-257
16 **Hsing AW**, McLaughlin JK, Olsen JH, Mellemkjar L, Wacholder S, Fraumeni JF Jr. Cancer risk following primary hemochromatosis: a population-based cohort study in Denmark. *Int J Cancer* 1995; **60**: 160-162
17 **Fracanzani AL**, Conte D, Fraquelli M, Taioli E, Mattioli M, Losco A, Fargion S. Increased cancer risk in a cohort of 230 patients with hereditary hemochromatosis in comparison to matched control patients with non-iron-related chronic liver disease. *Hepatology* 2001; **33**: 647-651
18 **Fargion S**, Stazi MA, Fracanzani AL, Mattioli M, Sampietro M, Tavazzi D, Bertelli C, Patriarca V, Mariani C, Fiorelli G. Mutations in the HFE gene and their interaction with exogenous risk factors in hepatocellular carcinoma. *Blood Cells Mol Dis* 2001; **27**: 505-511
19 **Hellerbrand C**, Poppl A, Hartmann A, Scholmerich J, Lock G. HFE C282Y heterozygosity in hepatocellular carcinoma: evidence for an increased prevalence. *Clin Gastroenterol Hepatol* 2003; **1**: 279-284
20 **Blanc JF**, De Ledinghen V, Bernard PH, de Verneuil H, Winnock M, Le Bail B, Carles J, Saric J, Balabaud C, Bioulac-Sage P. Increased incidence of HFE C282Y mutations in patients with iron overload and hepatocellular carcinoma developed in non-cirrhotic liver. *J Hepatol* 2000; **32**: 805-811
21 **Boige V**, Castera L, de Roux N, Ganne-Carrie N, Ducot B, Pelletier G, Beaugrand M, Buffet C. Lack of association between HFE gene mutations and hepatocellular carcinoma in patients with cirrhosis. *Gut* 2003; **52**: 1178-1181
22 **Cauza E**, Peck-Radosavljevic M, Ulrich-Pur H, Datz C, Gschwantler M, Schoniger-Hekele M, Hackl F, Polli C, Rasoul-Rockenschaub S, Muller C, Wrba F, Gangl A, Ferenci P. Mutations of the HFE gene in patients with hepatocellular carcinoma. *Am J Gastroenterol* 2003; **98**: 442-447
23 **Nahon P**, Sutton A, Rufat P, Ziol M, Thabut G, Schischmanoff PO, Vidaud D, Charnaux N, Couvert P, Ganne-Carrie N, Trinchet JC, Gattegno L, Beaugrand M. Liver iron, HFE gene mutations, and hepatocellular carcinoma occurrence in patients with cirrhosis. *Gastroenterology* 2008; **134**: 102-110
24 **Kowdley KV**. Iron, hemochromatosis, and hepatocellular carcinoma. *Gastroenterology* 2004; **127**: S79-S86
25 **Beutler E**. The significance of the 187G (H63D) mutation in hemochromatosis. *Am J Hum Genet* 1997; **61**: 762-764
26 **MacPhail AP**, Mandishona EM, Bloom PD, Paterson AC, Rouault TA, Gordeuk VR. Measurements of iron status and survival in African iron overload. *S Afr Med J* 1999; **89**: 966-972
27 **Mandishona E**, MacPhail AP, Gordeuk VR, Kedda MA, Paterson AC, Rouault TA, Kew MC. Dietary iron overload as a risk factor for hepatocellular carcinoma in Black Africans. *Hepatology* 1998; **27**: 1563-1566
28 **Turlin B**, Juguet F, Moirand R, Le Quilleuc D, Loreal O, Campion JP, Launois B, Ramee MP, Brissot P, Deugnier Y. Increased liver iron stores in patients with hepatocellular carcinoma developed on a noncirrhotic liver. *Hepatology* 1995; **22**: 446-450
29 **Borgna-Pignatti C**, Vergine G, Lombardo T, Cappellini MD, Cianciulli P, Maggio A, Renda D, Lai ME, Mandas A, Forni G, Piga A, Bisconte MG. Hepatocellular carcinoma in the thalassaemia syndromes. *Br J Haematol* 2004; **124**: 114-117
30 **Moyo VM**, Makunike R, Gangaidzo IT, Gordeuk VR, McLaren CE, Khumalo H, Saungweme T, Rouault T, Kiire CF. African iron overload and hepatocellular carcinoma (HA-7-0-080). *Eur J Haematol* 1998; **60**: 28-34
31 **Caldwell SH**, Crespo DM. The spectrum expanded: cryptogenic cirrhosis and the natural history of non-alcoholic fatty liver disease. *J Hepatol* 2004; **40**: 578-584
32 **Powell EE**, Cooksley WG, Hanson R, Searle J, Halliday JW, Powell LW. The natural history of nonalcoholic steatohepatitis: a follow-up study of forty-two patients for up to 21 years. *Hepatology* 1990; **11**: 74-80
33 **Fattovich G**, Stroffolini T, Zagni I, Donato F. Hepatocellular carcinoma in cirrhosis: incidence and risk factors. *Gastroenterology* 2004; **127**: S35-S50
34 **Adams LA**, Lymp JF, St Sauver J, Sanderson SO, Lindor KD, Feldstein A, Angulo P. The natural history of nonalcoholic fatty liver disease: a population-based cohort study. *Gastroenterology* 2005; **129**: 113-121
35 **Shimada M**, Hashimoto E, Taniai M, Hasegawa K, Okuda H, Hayashi N, Takasaki K, Ludwig J. Hepatocellular carcinoma in patients with non-alcoholic steatohepatitis. *J Hepatol* 2002; **37**: 154-160
36 **Hashimoto E**, Yatsuji S, Tobari M, Taniai M, Torii N, Tokushige K, Shiratori K. Hepatocellular carcinoma in patients with nonalcoholic steatohepatitis. *J Gastroenterol* 2009; **44** Suppl 19: 89-95
37 **Marrero JA**, Fontana RJ, Su GL, Conjeevaram HS, Emick DM, Lok AS. NAFLD may be a common underlying liver disease in patients with hepatocellular carcinoma in the United States. *Hepatology* 2002; **36**: 1349-1354
38 **Calle EE**, Rodriguez C, Walker-Thurmond K, Thun MJ. Overweight, obesity, and mortality from cancer in a prospectively studied cohort of U.S. adults. *N Engl J Med* 2003; **348**: 1625-1638
39 **Wolk A**, Gridley G, Svensson M, Nyren O, McLaughlin JK, Fraumeni JF, Adam HO. A prospective study of obesity and cancer risk (Sweden). *Cancer Causes Control* 2001; **12**: 13-21
40 **Polesel J**, Zucchetto A, Montella M, Dal Maso L, Crispo A, La Vecchia C, Serraino D, Franceschi S, Talamini R. The impact of obesity and diabetes mellitus on the risk of hepatocellular carcinoma. *Ann Oncol* 2009; **20**: 353-357
41 **Moller H**, Mellemgaard A, Lindvig K, Olsen JH. Obesity and cancer risk: a Danish record-linkage study. *Eur J Cancer* 1994; **30A**: 344-350
42 **Larsson SC**, Wolk A. Overweight, obesity and risk of liver cancer: a meta-analysis of cohort studies. *Br J Cancer* 2007; **97**: 1005-1008
43 **Caldwell SH**, Oelsner DH, Iezzoni JC, Hespenheide EE, Battle EH, Driscoll CJ. Cryptogenic cirrhosis: clinical characterization and risk factors for underlying disease. *Hepatology* 1999; **29**: 664-669
44 **Poonawala A**, Nair SP, Thuluvath PJ. Prevalence of obesity and diabetes in patients with cryptogenic cirrhosis: a case-control study. *Hepatology* 2000; **32**: 689-692
45 **Nair S**, Mason A, Eason J, Loss G, Perrillo RP. Is obesity an

independent risk factor for hepatocellular carcinoma in cirrhosis? *Hepatology* 2002; **36**: 150-155
46 **El-Serag HB**, Tran T, Everhart JE. Diabetes increases the risk of chronic liver disease and hepatocellular carcinoma. *Gastroenterology* 2004; **126**: 460-468
47 **El-Serag HB**, Hampel H, Javadi F. The association between diabetes and hepatocellular carcinoma: a systematic review of epidemiologic evidence. *Clin Gastroenterol Hepatol* 2006; **4**: 369-380
48 **Polesel J**, Talamini R, Montella M, Maso LD, Crovatto M, Parpinel M, Izzo F, Tommasi LG, Serraino D, La Vecchia C, Franceschi S. Nutrients intake and the risk of hepatocellular carcinoma in Italy. *Eur J Cancer* 2007; **43**: 2381-2387
49 **Yuan JM**, Gao YT, Ong CN, Ross RK, Yu MC. Prediagnostic level of serum retinol in relation to reduced risk of hepatocellular carcinoma. *J Natl Cancer Inst* 2006; **98**: 482-490
50 **Talamini R**, Polesel J, Montella M, Dal Maso L, Crispo A, Tommasi LG, Izzo F, Crovatto M, La Vecchia C, Franceschi S. Food groups and risk of hepatocellular carcinoma: A multicenter case-control study in Italy. *Int J Cancer* 2006; **119**: 2916-2921
51 **Sauvaget C**, Nagano J, Hayashi M, Spencer E, Shimizu Y, Allen N. Vegetables and fruit intake and cancer mortality in the Hiroshima/Nagasaki Life Span Study. *Br J Cancer* 2003; **88**: 689-694
52 **La Vecchia C**, Negri E, Decarli A, D'Avanzo B, Franceschi S. Risk factors for hepatocellular carcinoma in northern Italy. *Int J Cancer* 1988; **42**: 872-876
53 **Yu MW**, Hsieh HH, Pan WH, Yang CS, CHen CJ. Vegetable consumption, serum retinol level, and risk of hepatocellular carcinoma. *Cancer Res* 1995; **55**: 1301-1305
54 **Kuper H**, Tzonou A, Lagiou P, Mucci LA, Trichopoulos D, Stuver SO, Trichopoulou A. Diet and hepatocellular carcinoma: a case-control study in Greece. *Nutr Cancer* 2000; **38**: 6-12
55 **La Vecchia C**, Tavani A. Coffee and cancer risk: an update. *Eur J Cancer Prev* 2007; **16**: 385-389
56 **Gallus S**, Bertuzzi M, Tavani A, Bosetti C, Negri E, La Vecchia C, Lagiou P, Trichopoulos D. Does coffee protect against hepatocellular carcinoma? *Br J Cancer* 2002; **87**: 956-959
57 **Kono S**, Shinchi K, Imanishi K, Todoroki I, Hatsuse K. Coffee and serum gamma-glutamyltransferase: a study of self-defense officials in Japan. *Am J Epidemiol* 1994; **139**: 723-727
58 **Tanaka K**, Tokunaga S, Kono S, Tokudome S, Akamatsu T, Moriyama T, Zakouji H. Coffee consumption and decreased serum gamma-glutamyltransferase and aminotransferase activities among male alcohol drinkers. *Int J Epidemiol* 1998; **27**: 438-443
59 **Sharp DS**, Everhart JE, Benowitz NL. Coffee, alcohol, and the liver. *Ann Epidemiol* 1999; **9**: 391-393
60 **Shimazu T**, Tsubono Y, Kuriyama S, Ohmori K, Koizumi Y, Nishino Y, Shibuya D, Tsuji I. Coffee consumption and the risk of primary liver cancer: pooled analysis of two prospective studies in Japan. *Int J Cancer* 2005; **116**: 150-154
61 **Gelatti U**, Covolo L, Franceschini M, Pirali F, Tagger A, Ribero ML, Trevisi P, Martelli C, Nardi G, Donato F. Coffee consumption reduces the risk of hepatocellular carcinoma independently of its aetiology: a case-control study. *J Hepatol* 2005; **42**: 528-534
62 **Inoue M**, Yoshimi I, Sobue T, Tsugane S. Influence of coffee drinking on subsequent risk of hepatocellular carcinoma: a prospective study in Japan. *J Natl Cancer Inst* 2005; **97**: 293-300
63 **Kurozawa Y**, Ogimoto I, Shibata A, Nose T, Yoshimura T, Suzuki H, Sakata R, Fujita Y, Ichikawa S, Iwai N, Tamakoshi A. Coffee and risk of death from hepatocellular carcinoma in a large cohort study in Japan. *Br J Cancer* 2005; **93**: 607-610
64 **Montella M**, Polesel J, La Vecchia C, Dal Maso L, Crispo A, Crovatto M, Casarin P, Izzo F, Tommasi LG, Talamini R, Franceschi S. Coffee and tea consumption and risk of hepatocellular carcinoma in Italy. *Int J Cancer* 2007; **120**: 1555-1559
65 **Tanaka K**, Hara M, Sakamoto T, Higaki Y, Mizuta T, Eguchi Y, Yasutake T, Ozaki I, Yamamoto K, Onohara S, Kawazoe S, Shigematsu H, Koizumi S. Inverse association between coffee drinking and the risk of hepatocellular carcinoma: a case-control study in Japan. *Cancer Sci* 2007; **98**: 214-218
66 **Larsson SC**, Wolk A. Coffee consumption and risk of liver cancer: a meta-analysis. *Gastroenterology* 2007; **132**: 1740-1745
67 **Bravi F**, Bosetti C, Tavani A, Bagnardi V, Gallus S, Negri E, Franceschi S, La Vecchia C. Coffee drinking and hepatocellular carcinoma risk: a meta-analysis. *Hepatology* 2007; **46**: 430-435
68 **Donato F**, Tagger A, Gelatti U, Parrinello G, Boffetta P, Albertini A, Decarli A, Trevisi P, Ribero ML, Martelli C, Porru S, Nardi G. Alcohol and hepatocellular carcinoma: the effect of lifetime intake and hepatitis virus infections in men and women. *Am J Epidemiol* 2002; **155**: 323-331
69 **Corrao G**, Bagnardi V, Zambon A, La Vecchia C. A meta-analysis of alcohol consumption and the risk of 15 diseases. *Prev Med* 2004; **38**: 613-619
70 **Munaka M**, Kohshi K, Kawamoto T, Takasawa S, Nagata N, Itoh H, Oda S, Katoh T. Genetic polymorphisms of tobacco- and alcohol-related metabolizing enzymes and the risk of hepatocellular carcinoma. *J Cancer Res Clin Oncol* 2003; **129**: 355-360
71 **Covolo L**, Gelatti U, Talamini R, Garte S, Trevisi P, Franceschi S, Franceschini M, Barbone F, Tagger A, Ribero ML, Parrinello G, Donadon V, Nardi G, Donato F. Alcohol dehydrogenase 3, glutathione S-transferase M1 and T1 polymorphisms, alcohol consumption and hepatocellular carcinoma (Italy). *Cancer Causes Control* 2005; **16**: 831-838
72 **Mizoue T**, Tokui N, Nishisaka K, Nishisaka S, Ogimoto I, Ikeda M, Yoshimura T. Prospective study on the relation of cigarette smoking with cancer of the liver and stomach in an endemic region. *Int J Epidemiol* 2000; **29**: 232-237
73 **Yun YH**, Jung KW, Bae JM, Lee JS, Shin SA, Min Park S, Yoo T, Yul Huh B. Cigarette smoking and cancer incidence risk in adult men: National Health Insurance Corporation Study. *Cancer Detect Prev* 2005; **29**: 15-24
74 **Zhu K**, Moriarty C, Caplan LS, Levine RS. Cigarette smoking and primary liver cancer: a population-based case-control study in US men. *Cancer Causes Control* 2007; **18**: 315-321
75 **Jee SH**, Ohrr H, Sull JW, Samet JM. Cigarette smoking, alcohol drinking, hepatitis B, and risk for hepatocellular carcinoma in Korea. *J Natl Cancer Inst* 2004; **96**: 1851-1856
76 **Evans AA**, Chen G, Ross EA, Shen FM, Lin WY, London WT. Eight-year follow-up of the 90,000-person Haimen City cohort: I. Hepatocellular carcinoma mortality, risk factors, and gender differences. *Cancer Epidemiol Biomarkers Prev* 2002; **11**: 369-376
77 **Sun CA**, Wu DM, Lin CC, Lu SN, You SL, Wang LY, Wu MH, Chen CJ. Incidence and cofactors of hepatitis C virus-related hepatocellular carcinoma: a prospective study of 12,008 men in Taiwan. *Am J Epidemiol* 2003; **157**: 674-682
78 **Ogimoto I**, Shibata A, Kurozawa Y, Nose T, Yoshimura T, Suzuki H, Iwai N, Sakata R, Fujita Y, Ichikawa S, Fukuda K, Tamakoshi A. Risk of death due to hepatocellular carcinoma among smokers and ex-smokers. Univariate analysis of JACC study data. *Kurume Med J* 2004; **51**: 71-81
79 **Fujita Y**, Shibata A, Ogimoto I, Kurozawa Y, Nose T, Yoshimura T, Suzuki H, Iwai N, Sakata R, Ichikawa S, Tamakoshi A. The effect of interaction between hepatitis C virus and cigarette smoking on the risk of hepatocellular carcinoma. *Br J Cancer* 2006; **94**: 737-739
80 **Tajada M**, Nerín J, Ruiz MM, Sánchez-Dehesa M, Fabre E. Liver adenoma and focal nodular hyperplasia associated with oral contraceptives. *Eur J Contracept Reprod Health Care* 2001; **6**: 227-230
81 **Giannitrapani L**, Soresi M, La Spada E, Cervello M, D'Alessandro N, Montalto G. Sex hormones and risk of liver tumor. *Ann N Y Acad Sci* 2006; **1089**: 228-236
82 **Rosenberg L**. The risk of liver neoplasia in relation to combined oral contraceptive use. *Contraception* 1991; **43**: 643-652
83 **Korula J**, Yellin A, Kanel G, Campofiori G, Nichols P. Hepatocellular carcinoma coexisting with hepatic adenoma. Inci-

dental discovery after long-term oral contraceptive use. *West J Med* 1991; **155**: 416-418
84 **Tesluk H**, Lawrie J. Hepatocellular adenoma. Its transformation to carcinoma in a user of oral contraceptives. *Arch Pathol Lab Med* 1981; **105**: 296-299
85 **Gordon SC**, Reddy KR, Livingstone AS, Jeffers LJ, Schiff ER. Resolution of a contraceptive-steroid-induced hepatic adenoma with subsequent evolution into hepatocellular carcinoma. *Ann Intern Med* 1986; **105**: 547-549
86 **Gyorffy EJ**, Bredfeldt JE, Black WC. Transformation of hepatic cell adenoma to hepatocellular carcinoma due to oral contraceptive use. *Ann Intern Med* 1989; **110**: 489-490
87 **Ferrell LD**. Hepatocellular carcinoma arising in a focus of multilobular adenoma. A case report. *Am J Surg Pathol* 1993; **17**: 525-529
88 **Herman P**, Machado MA, Volpe P, Pugliese V, Vianna MR, Bacchella T, Machado MC, Pinotti HW. [Transformation of hepatic adenoma into hepatocellular carcinoma in patients with prolonged use of oral contraceptives] *Rev Hosp Clin Fac Med Sao Paulo* 1994; **49**: 30-33
89 **Foster JH**, Berman MM. The malignant transformation of liver cell adenomas. *Arch Surg* 1994; **129**: 712-717
90 **Perret AG**, Mosnier JF, Porcheron J, Cuilleron M, Berthoux P, Boucheron S, Audigier JC. Role of oral contraceptives in the growth of a multilobular adenoma associated with a hepatocellular carcinoma in a young woman. *J Hepatol* 1996; **25**: 976-979
91 **Ito M**, Sasaki M, Wen CY, Nakashima M, Ueki T, Ishibashi H, Yano M, Kage M, Kojiro M. Liver cell adenoma with malignant transformation: a case report. *World J Gastroenterol* 2003; **9**: 2379-2381
92 **Closset J**, Veys I, Peny MO, Braude P, Van Gansbeke D, Lambilliotte JP, Gelin M. Retrospective analysis of 29 patients surgically treated for hepatocellular adenoma or focal nodular hyperplasia. *Hepatogastroenterology* 2000; **47**: 1382-1384
93 **Micchelli ST**, Vivekanandan P, Boitnott JK, Pawlik TM, Choti MA, Torbenson M. Malignant transformation of hepatic adenomas. *Mod Pathol* 2008; **21**: 491-497
94 **Zucman-Rossi J**, Jeannot E, Nhieu JT, Scoazec JY, Guettier C, Rebouissou S, Bacq Y, Leteurtre E, Paradis V, Michalak S, Wendum D, Chiche L, Fabre M, Mellottee L, Laurent C, Partensky C, Castaing D, Zafrani ES, Laurent-Puig P, Balabaud C, Bioulac-Sage P. Genotype-phenotype correlation in hepatocellular adenoma: new classification and relationship with HCC. *Hepatology* 2006; **43**: 515-524
95 **Maheshwari S**, Sarraj A, Kramer J, El-Serag HB. Oral contraception and the risk of hepatocellular carcinoma. *J Hepatol* 2007; **47**: 506-513
96 **Tsai JF**, Chuang LY, Jeng JE, Ho MS, Hsieh MY, Lin ZY, Wang LY. Betel quid chewing as a risk factor for hepatocellular carcinoma: a case-control study. *Br J Cancer* 2001; **84**: 709-713
97 **Bhide SV**, Shivapurkar NM, Gothoskar SV, Ranadive KJ. Carcinogenicity of betel quid ingredients: feeding mice with aqueous extract and the polyphenol fraction of betel nut. *Br J Cancer* 1979; **40**: 922-926
98 **Prokopczyk B**, Rivenson A, Bertinato P, Brunnemann KD, Hoffmann D. 3-(Methylnitrosamino)propionitrile: occurrence in saliva of betel quid chewers, carcinogenicity, and DNA methylation in F344 rats. *Cancer Res* 1987; **47**: 467-471
99 **Jeng JH**, Chang MC, Hahn LJ. Role of areca nut in betel quid-associated chemical carcinogenesis: current awareness and future perspectives. *Oral Oncol* 2001; **37**: 477-492
100 **Ramchandani AG**, D'Souza AV, Borges AM, Bhisey RA. Evaluation of carcinogenic/co-carcinogenic activity of a common chewing product, pan masala, in mouse skin, stomach and esophagus. *Int J Cancer* 1998; **75**: 225-232
101 **Stich HF**, Anders F. The involvement of reactive oxygen species in oral cancers of betel quid/tobacco chewers. *Mutat Res* 1989; **214**: 47-61
102 **Wang CK**, Su HY, Lii CK. Chemical composition and toxicity of Taiwanese betel quid extract. *Food Chem Toxicol* 1999; **37**: 135-144
103 **Tsai JF**, Jeng JE, Chuang LY, Ho MS, Ko YC, Lin ZY, Hsieh MY, Chen SC, Chuang WL, Wang LY, Yu ML, Dai CY, Ho C. Habitual betel quid chewing as a risk factor for cirrhosis: a case-control study. *Medicine* (Baltimore) 2003; **82**: 365-372
104 **Tsai JF**, Jeng JE, Chuang LY, Ho MS, Ko YC, Lin ZY, Hsieh MY, Chen SC, Chuang WL, Wang LY, Yu ML, Dai CY. Habitual betel quid chewing and risk for hepatocellular carcinoma complicating cirrhosis. *Medicine* (Baltimore) 2004; **83**: 176-187
105 **Kirk GD**, Bah E, Montesano R. Molecular epidemiology of human liver cancer: insights into etiology, pathogenesis and prevention from The Gambia, West Africa. *Carcinogenesis* 2006; **27**: 2070-2082
106 **Aguilar F**, Hussain SP, Cerutti P. Aflatoxin B1 induces the transversion of G-->T in codon 249 of the p53 tumor suppressor gene in human hepatocytes. *Proc Natl Acad Sci USA* 1993; **90**: 8586-8590
107 **Deng ZL**, Ma Y. Aflatoxin sufferer and p53 gene mutation in hepatocellular carcinoma. *World J Gastroenterol* 1998; **4**: 28-29
108 **Bressac B**, Kew M, Wands J, Ozturk M. Selective G to T mutations of p53 gene in hepatocellular carcinoma from southern Africa. *Nature* 1991; **350**: 429-431
109 **Coursaget P**, Depril N, Chabaud M, Nandi R, Mayelo V, LeCann P, Yvonnet B. High prevalence of mutations at codon 249 of the p53 gene in hepatocellular carcinomas from Senegal. *Br J Cancer* 1993; **67**: 1395-1397
110 **Hsu IC**, Metcalf RA, Sun T, Welsh JA, Wang NJ, Harris CC. Mutational hotspot in the p53 gene in human hepatocellular carcinomas. *Nature* 1991; **350**: 427-428
111 **Ming L**, Thorgeirsson SS, Gail MH, Lu P, Harris CC, Wang N, Shao Y, Wu Z, Liu G, Wang X, Sun Z. Dominant role of hepatitis B virus and cofactor role of aflatoxin in hepatocarcinogenesis in Qidong, China. *Hepatology* 2002; **36**: 1214-1220
112 **Aguilar F**, Harris CC, Sun T, Hollstein M, Cerutti P. Geographic variation of p53 mutational profile in nonmalignant human liver. *Science* 1994; **264**: 1317-1319
113 **Challen C**, Lunec J, Warren W, Collier J, Bassendine MF. Analysis of the p53 tumor-suppressor gene in hepatocellular carcinomas from Britain. *Hepatology* 1992; **16**: 1362-1366
114 **Zhang YJ**, Rossner P Jr, Chen Y, Agrawal M, Wang Q, Wang L, Ahsan H, Yu MW, Lee PH, Santella RM. Aflatoxin B1 and polycyclic aromatic hydrocarbon adducts, p53 mutations and p16 methylation in liver tissue and plasma of hepatocellular carcinoma patients. *Int J Cancer* 2006; **119**: 985-991
115 **Qian GS**, Ross RK, Yu MC, Yuan JM, Gao YT, Henderson BE, Wogan GN, Groopman JD. A follow-up study of urinary markers of aflatoxin exposure and liver cancer risk in Shanghai, People's Republic of China. *Cancer Epidemiol Biomarkers Prev* 1994; **3**: 3-10
116 **Chen CJ**, Wang LY, Lu SN, Wu MH, You SL, Zhang YJ, Wang LW, Santella RM. Elevated aflatoxin exposure and increased risk of hepatocellular carcinoma. *Hepatology* 1996; **24**: 38-42

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EDITORIAL

Primary biliary cirrhosis: What do autoantibodies tell us?

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Abstract

Primary biliary cirrhosis (PBC) is a chronic, progressive, cholestatic, organ-specific autoimmune disease of unknown etiology. It predominantly affects middle-aged women, and is characterized by autoimmune-mediated destruction of small- and medium-size intrahepatic bile ducts, portal inflammation and progressive scarring, which without proper treatment can ultimately lead to fibrosis and hepatic failure. Serum autoantibodies are crucial tools for differential diagnosis of PBC. While it is currently accepted that antimitochondrial antibodies are the most important serological markers of PBC, during the last five decades more than sixty autoantibodies have been explored in these patients, some of which had previously been thought to be specific for other autoimmune diseases.

Key words: Primary biliary cirrhosis; Autoimmune disease; Autoantibody; Anti-mitochondrial antibody; Anti-gp210 antibody; Anti-sp100 antibody; Anti-centromere antibodies

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INTRODUCTION

Primary biliary cirrhosis (PBC) is a progressive autoimmune liver disease characterized by infiltration of lymphocytes in portal tracts, progressive destruction of intrahepatic small bile ducts and the presence of serum antimitochondrial antibodies (AMA)[1,2]. As is the case for the majority of autoimmune diseases, PBC affects predominantly women. Recent investigations have suggested that PBC, sometimes asymptomatic, is not a rare disease. During the last several years advanced biochemical assays, further delineation of specific liver histological findings, more effective serum autoantibody detection methods and improved diagnostic abilities have led to higher prevalence estimates worldwide[3-5]. Currently it is believed that PBC is likely to be triggered by a combination of environmental factors including infection in a genetically susceptible individual. This hypothesis is supported by the high concordance rate of PBC among first-degree relatives and in homozygous twins (approximately 60%)[6,7]. Specific immunologic damage to biliary epithelium and a mechanism of tissue destruction in PBC has been elucidated[8,9]. In addition, epitopes of T cells and B cells targeting mitochondrial autoantigens have been identified[10-12]. Furthermore, a number of autoantibodies previously thought to be spe-

cific markers for another autoimmune disease have been detected in patients with PBC.

Disease progression and clinical manifestations in PBC varies. The fact that a variety of autoantibodies have been detected in PBC suggests the disease has a complicated pathogenesis. In this review, the properties of these autoantibodies and their autoantigen characteristics, as well as their pathogenetic and clinical significance were discussed.

AMA

The presence of AMA is pathognomonic for PBC[13], and it is generally accepted that AMA can be detected in serum years before the advent of any clinical manifestation or biochemical abnormality[14-16]. AMA were first described in 1958[17] in sera from patients with chronic liver disorders and then detected by Walker *et al*[18] in 1965 using an immunofluorescence test. In the past 40 years an enormous number of experimental studies have focused on AMA, and numerous rewarding discoveries have been made. There are nine subtypes of AMA, four of which have been involved in PBC, including anti-M2, anti-M4, anti-M8 and anti-M9. It has been demonstrated that the autoantigens recognized by anti-M2 are located in the inner membranes of mitochondria, whereas those recognized by anti-M4, anti-M8 and anti-M9 are located in the outer mitochondrial membranes. Anti-M9 can be detected in both anti-M2-positive and -negative PBC patients, while anti-M4 is only positive in the presence of anti-M2. All four of these AMA subtypes are relatively specific for the diagnosis of PBC.

Anti-M2

M2 has been found to contain five antigenic determinants, with molecular weights of 70 kDa (a), 56 kDa (b), 51 kDa (c) 45 kDa (d) and 36 kDa (e), all of which were identified subsequently as members of the 2-oxoacid dehydrogenase complex of enzymes within the mitochondrial respiratory chain, including the E2 subunit of the pyruvate dehydrogenase complex (PDC-E2), the E2 subunit of the branched-chain 2-oxoacid dehydrogenase complex, the E2 subunit of the 2-oxoglutarate dehydrogenase complex, E1t alfa subunits of PDC and E3 binding protein (protein X)[19,20]. The exact molecular weight of the M2 band differs among laboratories according to mitochondria species being used and specifics of techniques for antigen preparation and detection. In patients with PBC, approximately 90%-95% of serum samples react against PDC-E2, making this the most important autoantigen in the disease. Anti-M2 is the most important subtype used in routine diagnostic tests for PBC. Its level in affected sera is high and it also exists in other body fluids such as saliva and bile[21-23]. As AMA is considered to be the hallmark of PBC, a positive test is potentially diagnostic, or at least indicative that the individual is at increased risk for future development of PBC[15].

Anti-M4

The anti-M4 antibody was originally detected in patients with chronic cholestatic liver disease (mixed form) associated with two different types of complement-fixing AMA[24]. M4 is a single antigen with molecular weight of 52 kDa. It can be detected by a complement fixation test but not immunoblotting. Unlike M2, the M4 antigen is trypsin-insensitive and its band at sucrose densities is 1.08 to 1.14. Anti-M4 is found predominantly in patients with histological features of chronic active hepatitis and PBC. Recent studies have identified the major proteins in the M4 fraction which is related to the PDC-E1 subunits and sulphite oxidase[25,26].

Anti-M8

The M8 antigen is also trypsin-sensitive with a band at sucrose densities of 1.16 to 1.24. Anti-M8 has been found only in coexistence with anti-M2, the presence of anti-M8 indicates progressive disease activity. On the other hand, not all anti-M2-positive patients have anti-M8. Like M4, the M8 antigen also locates in the outer mitochondrial membranes[27].

Anti-M9

Anti-M9 antibody was accidentally found when testing anti-M2-positive sera against trypsinized submitochondrial particles from rat liver shown to be devoid of anti-M2[28]. Anti-M9 antibody is detected predominantly in patients with asymptomatic and early PBC, and it also can be positive in anti-M2-negative PBC patients. Unlike anti-M4 and anti-M8, which seem to reflect disease activity, anti-M9 antibody occurs early in PBC. Patients with only anti-M9 have all the typical biochemical features found in classic anti-M2-positive patients, but seem to have slower disease progression and benign outcome, whereas patients having complement-fixing antibodies against anti-M2, anti-M4, and anti-M8 seem to have more active disease and worse outcome[29-31], though this finding wasn't supported by a blinded study on Dutch PBC patients conducted by Vleggaar *et al*[32].

ROLE OF AMA IN PBC PATHOGENESIS

Although AMA serve as highly sensitive markers for the diagnosis of PBC, autoantibodies against various mitochondrial enzymes can frequently be detected in patients with other diseases, such as primary Sjögren's syndrome (pSS), scleroderma, autoimmune hepatitis[33,34] and some infectious diseases like tuberculosis and viral hepatitis[35-38]. It is very interesting that the prevalence of AMA in first-degree relatives of PBC probands is as high as 13.1%, whereas in gender, age, race, and residence-matched controls the prevalence is only 1%, suggesting that environmental risks and genetic determinants are likely implicated in the etiology of PBC[7].

As no clinical correlation can be found, and animal models with serum AMA do not consistently have PBC-like liver lesions, the exact role played by AMA in the immunopathology and pathogenesis of PBC remains elusive. However, current data indicate that the destruction

of biliary cells is mediated by liver-infiltrating autoreactive T cells specific for the dominant PDC-E2 autoantigen[39]. The dominant epitopes of autoreactive T and B cells have been identified. The $CD4^+$ T cell epitope appears to localize to peptides 163-176, the $CD8^+$ T cell epitope appears to localize to peptides 159-167, while the B cell epitope appears to localize to peptides 167-186[39-43]. Furthermore, the most prominent immune features of autoreactive $CD4^+$ and $CD8^+$ T cells can be detected in peripheral blood from patients with PBC. The disease-related AMA-specific $CD8^+$ T cells are enriched up to about 10-fold and the $CD4^+$ T cells are enriched up to more than 100-fold in liver compared to peripheral blood samples[42,44]. Presently the data suggest that B and T cells in PBC patients respond simultaneously to the same autoantigen, and that both are involved in the pathogenesis of PBC.

Study of stored sera of well-characterized PBC patients followed for 7-28 years indicate that AMA levels are not associated with disease severity and progression. Most studies except the one conducted by Poupon *et al*[45] did not support that AMA levels could be affected by treatments during the observation period[45-47]. In fact, low levels of AMA persisted for up to 11 years following liver transplantation[47]. AMA are non-organ- and non-species-specific, and contain IgA, IgG and IgM subclasses. Data from PBC patients demonstrate that the presence of AMA IgA in sera or saliva might be associated with disease progression[23] and some studies suggested that greater concentrations of AMA IgA in biliary and mucosal secretions, constant transcytosis, would render the exposed cells more susceptible to apoptosis resulting in subsequent bile duct damage[48], while others proposed the hypothesis that AMA IgA can be transported to the vascular side of the bile duct cell where it can induce apoptosis by reacting with PDC-E2-like molecules located on the luminal surface cell membrane[49]. Many studies have demonstrated that the different AMA IgG subclasses have different clinical significance. PBC patients positive for IgG3 AMA had histologically more advanced disease and were more frequently cirrhotic than those who were negative. Furthermore, there was a positive correlation between AMA IgG3 titers and Mayo risk scores: this subclass is associated with poor prognosis, possibly reflecting the peculiar ability of this isotype to engage mediators of immunological damage[50].

Currently it is believed that a positive AMA titer is virtually pathognomonic of current PBC or risk for future development of the disorder, although the mechanisms leading to the generation of AMA have not been elucidated. Several possible mechanisms have been suggested regarding the generation of AMA, such as oxidative damage, molecular mimicry and changed biliary epithelial cell (BEC) apoptosis[51,52]. The fact that high levels of AMA can be detected in patients with acute liver failure supports the hypothesis that oxidative stress-induced liver damage may lead to induction of AMA[53]. But it is also surprisingly true that the AMA in these patients disappear rapidly, suggesting the pathogenesis of PBC is multifactorial. It has been demonstrated that molecular mimicry between bacterial or viral antigens and mitochondrial antigens can trigger the generation of AMA in PBC[54,55]. Modification of the inner lipoyl domain of E2 with halide or ethyl halide results in increased reactivity of AMA from PBC patients, suggesting that xenobiotics might make cellular components antigenic[56].

There is growing evidence showing that the onset of PBC may be the result of inefficient removal of apoptotic cells. It is of interest to note that a recent report proposed that PDC-E2 in patients with PBC is released without caspase cleavage from apoptotic BEC, supported by the fact that glutathionylation of the lysine lipoic acid moiety on the PDC-E2 is sometimes, though not commonly, decreased by serum AMA *via* Bcl-2[57]. Other studies show that apoptotic cells are phagocytosed by BECs, a function mediated by anti-CD16, and so consequently act as an exogenous source of autoantigens in cholangiocytes[9,58]. Defects in the elimination of apoptotic cells can lead to secondary necrosis accompanied by subsequent release of intracellular components, which might explain the generation of autoantibodies against intracellular antigens like AMA[59].

Further studies in the field of AMA in PBC have led to speculation about the existence of an AMA-negative PBC subgroup. It is not clear whether there is indeed such a subgroup, having distinct features, or if this is an artifact due to technical limitations of current AMA detection methods leading to false-negative results in some PBC patients[60]. Present data indicate that there is no discernable difference between AMA-positive and -negative PBC in terms of clinical manifestations, liver biochemistry and histopathology findings, disease course, as well as response to treatment[61-63]. As more sensitive and specific serologic tests are applied, many patients initially believed to be AMA-negative are subsequently found to be AMA-positive[64,65]. These findings cast doubt on the existence of a true AMA-negative PBC subgroup.

ANTINUCLEAR ANTIBODIES IN PBC

Antinuclear dot antibodies (SP100, PML, NDP52 and SP140)

PBC patients often have autoantibodies with nuclear dot (ND) stain patterns in the indirect immunofluorescence (IIF) assay. The major antigens associated with ND are as follows: sp100 proteins, which are transcription-activating proteins autoantigenic primarily in patients with PBC and occasionally in rheumatic disorders[66,67]; promyelocytic leukemia (PML) protein, a transformation and cell-growth suppressing protein aberrantly expressed in PML cells that was discovered in studies of the development of acute PML; NDP52, a protein of the myosin VI binding partners which was previously shown to contribute to innate immunity[68,69]; and sp140 proteins, which are identified as autoantigenic proteins in PBC recently. Sp100 and PML were discovered in the context of leukemic transformation and as autoantigens in PBC[70].

They are reported to be co-autoimmunogenic, often in patients with PBC[71]. The sp100 antigen was described by Szostecki *et al*[66] as a peptide of 480 amino acids with an aberrant electrophoretic mobility to 100 kDa, and a calculated molecular weight of 53 kDa. It was subsequently characterized by complementary DNA cloning, and the deduced amino acid sequence was found to contain sequence similarities with HIV-1 nef proteins[72]. The prevalence of anti-sp100 antibodies in PBC is about 25%, and it appears to be highly specific for a diagnosis of PBC, but only when other diseases can be excluded and the typical clinical context is present[73,74]. The presence of anti-sp100 antibodies serves as a serologic marker of PBC, which could be useful in clinics to confirm the diagnosis, especially in AMA-negative PBC patients[75,76]. Recent data indicate that as reports of AMA-negative PBC decrease due to development of more sensitive and specific serologic tests for serum AMA, anti-sp100 antibodies appear to be more common in AMA-positive PBC patients than in those who are AMA-negative[77,78]. Also, anti-sp100 antibodies are increasingly found to be present in many clinical conditions, such as systemic lupus erythematosus (SLE) and pSS. It is of interest to note that among female patients with urinary tract infections but no liver disease, 80% of the AMA-positive, but none of the AMA-negative patients were also positive for anti-sp100 antibodies. It is also well established that among PBC patients, about 74% of patients with urinary tract infections were positive for anti-sp100, whereas the positivity was only 4.8% in PBC patients without urinary tract infections[79]. Given the high specificity of anti-sp100 as an immunoserological hallmark of PBC, these findings support the hypothesis that some infections such as *Escherichia coli* are involved in the induction of PBC-specific autoimmunity.

PML protein was discovered in cells of patients with acute PML as a protein fused with the retinoic acid receptor-a (RAR)[80,81]. PML protein functions as a nuclear hormone receptor transcriptional coactivator[82]. Subsequently it was shown to form ND patterns when tested by immunofluorescence microscopy with serum anti-PML antibodies from PBC patients. Anti-PML antibodies often coexist with anti-sp100 antibodies in individuals with PBC[71], and are present in about 19% of PBC patients[83]. Current study indicates that anti-PML antibodies are highly specific for PBC even when autoantibodies against mitochondrial antigens are not found[84].

Anti-sp140 antibodies were recently identified for the first time in patients with PBC by Granito *et al*[85]. They are present in about 15% of PBC patients and are highly specific for PBC. Anti-sp140 antibodies coexist with anti-sp100 and anti-PML antibodies. No association was found between anti-sp140 and any particular clinical feature of PBC.

Antinuclear pore antibodies (gp210 and p62)

In addition to AMA, a number of nuclear antigens have been recognized as targets of antinuclear antibodies (ANA) in patients with PBC, including several components of the nuclear pore complex (NPC), such as the gp210 and p62 proteins. These antibodies have a nuclear periphery fluorescence pattern in the IIF assay, as first reported by Ruffatti *et al*[86] in 1985. Several reports revealed that the frequency of PBC-specific nuclear envelope antibodies ranged from 16% to 30%[76,87], and that the frequency increased greatly when fluorescent-labeled specific antiserum of the IgG subclass was applied[88,89]. In 1990 a study by Lassoued *et al*[90] showed that autoantibodies from patients with PBC having a punctate fluorescence pattern in IIF react with a protein of molecular mass approximately 200 kDa, which was identified as the NPC membrane protein gp210[91]. Gp210 is an integral glycoprotein of the nuclear pore consisting of three main domains: a large glycosylated luminal domain, a single hydrophobic transmembrane segment and a short cytoplasmic tail. Gp210 is recognized by antibodies in approximately 25% of patients with PBC[92]. The gp210 epitope recognized by most of the autoantibodies is a 15 amino acid stretch in the cytoplasmic, carboxyl-terminal domain of the protein. In the ANA category, these anti-gp210 antibodies are particularly significant since they are highly specific for PBC[93,94]. In addition, several reports link the presence of anti-gp210 antibodies in PBC patients with disease severity and poor prognosis. Since the presence of anti-gp210 antibodies correlates with an unfavorable disease course and more rapid progression, it is useful for monitoring the effect of ursodeoxycholic acid and for the early identification of patients at high risk for end-stage hepatic failure, and so may potentially become an important prognostic marker in PBC patients[95,96]. Findings to date clearly indicate that anti-gp210 antibodies having the best predictive value regarding progression to end-stage hepatic failure. The proposed mechanism for this predictive role is based on the following hypothesis that the breakdown of immunological tolerance to mitochondrial antigens such as PDC-E2 is not enough for the progression to hepatic failure, whereas the breakdown of immunological tolerance to nuclear antigens such as gp210, in which molecular mimicry is involved as well as increased and aberrant expression of gp210 in small bile ducts, may play a crucial role[97].

A few years after the discovery of anti-gp210 antibodies in PBC, reactivity of PBC sera with a 60 kDa component of NPC was reported. Anti-p62 antibodies, which also generate a perinuclear pattern in IIF, were first described in 1987[98-100]. They occur as frequently as the anti-gp210 glycoprotein autoantibodies[101], and with a specificity for PBC of up to 97%. Anti-p62 antibodies reacting with the 60 kDa component localize to the NPC. The frequency of anti-p62 antibodies in PBC is about 30%-55%. Their presence in PBC is not associated with AMA, but is associated with disease progression. Data from a multicenter study indicated that anti-p62 complex antibodies might be related to the progressive or advanced stage of PBC[99,102], that their prevalence is higher in symptomatic patients and that they are associated with more severe

Table 1 Autoantibodies in primary biliary cirrhosis that are closely related to other autoimmune diseases

No.	Autoantibody	Autoantigen properties	Prevalence in PBC (%)	Clinical associations	Ref.
ANA					
1	Anti-chromatin	Chromatin	8.9-25.0	Anti-chromatin antibodies are reported to be associated with disease activity in AIH, but their roles in PBC remains to be investigated	[99,127-129]
2	Anti-dsDNA	Double-stranded deoxyribonucleic acid	17.0-22.0	Anti-dsDNA antibodies are one of important criteria for the diagnosis of SLE. Co-existence of AMA and anti-dsDNA autoantibodies can be considered the serological profile of AIH/PBC overlap syndrome	[76,127,128,130,131]
3	Anti-ssDNA	Single-stranded deoxyribonucleic acid	59.0-71.0	Anti-ssDNA antibodies can be detected in many diseases	[132,133]
4	Anti-histone	Histone	43.6	Anti-histone antibodies are generally considered to be related to drug-induced lupus, though it can be detected in many autoimmune diseases including PBC	[132,134]
5	Anti-scl-70	Topoisomerase-1	3.0-24.0	Anti-scl-70 antibody serve as a specific maker for diffuse SSc and presents in 30%-60% of subjects with diffuse SSc	[127,132]
6	Anti-Sm	Proteins of 28/29, 16, 16.5, 18, and 12, 11, 6 kDa which participate in pre-messenger RNA processing into spliced mature mRNA	7.0-34.0	Anti-Sm autoantibodies are highly specific for SLE	[76,127,132,135]
7	Anti-SSA	Intracellular ribonucleoproteins of 60 and 52 kDa that are associated with small RNAs	5.0-30.0	Anti-Ro(SS-A) and anti-La(SS-B) antibodies are more frequently seen in SS and SLE. Their presence in PBC suggests that PBC often overlaps with SS	[76,127,132,135,136]
8	Anti-SSB	An intracellular ribonucleoprotein of 47 kDa that are associated with small RNAs	2.0-21.0		
9	Anti-RNP	Ribonucleoprotein	5.0	More frequently seen in SLE	[76,127]
10	Anti-Jo-1	Histidyl tRNA synthetase	26.0	Anti-Jo-1 antibodies are predominantly detected in patients with myositis	[135]
11	Anti-U1RNP	U1snRNPs that contain specific proteins of 70, 33 and 20 kDa	3.1-5.0	Anti-U1snRNP antibodies predominantly present in SLE, and can be detected in PBC patients. The clinical significance of anti-U1snRNP antibodies in PBC is unknown	[137,138]
Other liver diseases-associated autoantibodies					
12	Anti-SMA	A variety of target antigens including F-actin, G-actin, myosin, tropomyosin, troponin, desmin, vimentin, keratin, *etc.*	8.0-25.0	Anti-SMAs present mainly in AIH-Ⅰ, and can also be detected in chronic active hepatitis. The presences of anti-SMAs in PBC are potential indicators of AIH/PBC overlap syndrome	[131,139]
13	Anti-SLA	SLA and liver and pancreas antigen	2.0-3.9	Anti-SLAs are autoantibodies seen in AIH-Ⅲ.The presences of SLA autoantibodies in PBC indicate secondary autoimmune hepatitis	[9,140,141]
14	Anti-LKM	Liver kidney microsomal antigen	0.7	Anti-LKM antibodies occur preferentially in AIH-Ⅱ. Anti-LKM autoantibodies can be seen in 21.4% of HCV-infected PBC patients, which suggests a close association between LKM and HCV-infected PBC	[142]
15	Anti-ASGPR	Asialoglycoprotein receptor	22.0-23.0	Anti-ASGPR antibodies mainly present in AIH and PBC. The autoimmune responses against ASGPR have been implicated in the development of AIH and PBC	[143-146]
16	Anti-LCM	Liver cell membrane specific antigen	42.0	Anti-LCM antibodies are detected predominantly in patients with HBsAg-negative chronic active hepatitis, but are also found in other liver diseases such as PBC	[147-149]
17	Anti-LSP	Liver specific protein	48.5	Anti-LSP antibodies present in viral hepatitis and autoimmune liver disease, and are found to correlate with severity of periportal inflammation and piecemeal necrosis in PBC	[144,150]
18	Anti-calreticulin	Calreticulin	20.0	Anti-calreticulin antibodies present in autoimmune liver disorder and IBD. They are not specific for PBC	[151,152]
19	Anti-FH	Fumarate hydratase	19.4	Anti-FH antibodies are found to be present predominantly in AIH. It can also be detected in PBC and other liver disease. The prevalence and clinic significance of anti-FH in PBC need further study	[153]
20	Anti-PGAM-B	Phosphoglycerate mutase isozyme B	16.7	Anti-PGAM-B antibodies are found to be present in 70.0% of AIH and 16.7% of PBC. It is also present in about 10% of viral hepatitis and 3.7% of healthy control. The clinical significance of anti-FH needs further study	[154]

21	Anti-p97/VCP	P97/valosin-containing protein	12.5	Anti-p97/VCP antibodies predominantly present in PBC, and can be detected in about 9.7% of AIH. The presence of anti-p97/VCP antibodies in PBC suggests less progressive disease course and benign prognosis	[155-157]
22	Anti-GSTA1-1	Glutathione S-transferase	10.0	Anti-GST autoantibodies are detected in 16.0% of AIH and 10.0% of PBC. Patients of AIH with positive anti-GST have severe diseases and poor prognosis	[158]
23	Anti-ASL	Argininosuccinate lyase	23.0	Anti-argininosuccinate lyase is a newly identified autoantibody in liver disease and its clinical relevance remains unknown	[159]
24	Anti-calmodulin	Calmodulin	IgM 50.0 IgA 42.9	Anti-calmodulin autoantibodies neither associate with anti-SMA, ANA and AMA, nor with hyperglobulinemia. The clinic significant of anti-calmodulin is unclear	[160]
Gastroenteropathy-associated autoantibodies					
25	ASCA	Baker's yeast saccharomyces cerevisiae	24.2	ASCA serves as a serological marker of Crohn's disease, and has also been detected in other autoimmune disorders and in 5%-6.3% of blood donors. The prevalence of ASCA in AIH is 20%-30%, in AMA-negative PBC 44%. ASCA is common in PBC patients and correlates with higher level of circulating IgA. The prevalence of ASCA in PBC may be an indirect sign of enhanced mucosal immunity, but does not necessarily indicate concomitant inflammatory bowel disease	[161-163]
26	Anti-Galectin-3	Galectin-3, a member of -galactoside-binding lectins	30.0	Anti-Galectin-3 autoantibodies are primarily associated with Crohn's disease, and correlate negatively with disease activity. The significance of anti-Galectin-3 IgG autoantibodies in patients with PBC is unknown	[164]
27	Anti-tTG	Tissue transglutaminase	10.0-26.7	Anti-tTG autoantibody is mainly found in celiac disease. The prevalence of anti-tTG in PBC varies due to different types of substrate utilized in detection	[127,165,166]
28	AGA	Gliadin	16.0-21.0	Anti-gliadin antibodies are considered as the most reliable serological markers for celiac disease. They are also frequently seen in PBC, and IgA subclass of anti-gliadin antibodies are more pronounced in patients with Scheuer's stage Ⅲ-Ⅳ disease	[166,167]
Vasculitis-associated autoantibodies					
29	ANCA	Antigens including proteinase 3, myeloperoxidase, bactericidal/permeability-increasing protein, lactoferrin,human leukocyte elastase, cathepsin G, lysozyme, azurocidin, *etc.*	2-26	ANCAs are primarily associated with systemic vasculitides such as Wegener's granulomatosis, microscopic polyangiitis and Churg-Strauss syndrome	[76,131,168]
30	Anti-MPO	Myeloperoxidase	9.0	Predominantly in microscopic polyangiitis, necrotizing and crescentic glomerulonephritis, Churg-Strauss syndrome	[169]
31	Anti-PR3	Proteinase 3	3.0	Predominantly in Wegener's granulomatosis, and also detectable in microscopic polyangiitis, necrotizing and crescentic glomerulonephritis	[127]
32	Anti-LF	Lactoferrin	25.0-35.7	Are detected in several autoimmune disorders, such as Crohn's disease, SLE, systemic vasculitides. They are not specific markers for PBC	[170,171]
Thrombophilia-associated autoantibodies					
33	Anti-β2GP Ⅰ	β(2)-glycoprotein Ⅰ	IgG 2-15	Represent specific features of patients with antiphospholipid syndrome. Their presence in PBC often indicates severe disease and worse prognosis	[127,172,173]
34	Acl	Cardiolipin	IgG 27.3		
35	Anti-PS	Phosphatidylserine	IgM 75		
36	Anti-PT	Prothrombin	IgG 7		
37	Anti-PE	Phosphatydilethanolamine	IgG 5		
Diabetes mellitus-associated autoantibodies					
38	Anti-GAD	Glutamic acid decarboxylase	5.5	Anti-GAD occurs preferentially in the patients with type 1 diabetes. Clinical significance of Anti-GAD in PBC is unclear	[174]
39	Anti-SOX13	Transcriptional factor SOX13	18.0	SOX13 was initially identified in type 1 diabetes. The present of anti-SOX13 in PBC may merely indicate an immune response to products of damage to parenchymal tissue	[175]
Autoimmune thyroid diseases-associated autoantibodies					
40	Anti-TG	Thyroglobulin	54.5	Anti-TG, anti-TPO and anti-TR are markers of autoimmune thyroid diseases. Their significances in PBC are unknown	[176]
41	Anti-TPO	Thyroid peroxidase	45.5		
42	Anti-TR	TSH receptor	9.1		

Others autoantibodies					
43	Anti-CCP	Cyclic citrullinated peptide	2.7-4.0	Anti-CCP antibodies are highly specific for RA with sensitivity of 60%-70%. Presence of anti-CCP antibodies in PBC patients suggests RA overlap	[125,126,177]
44	Anti-ClpP	Microbial caseinolytic proteases P	30-47	ClpP is highly conserved among bacteria. Anti-ClpP in PBC suggests infection factors and molecular mimicry involved in the pathogenesis	[178,179]
45	Anti-β-subunit of bacterial RNA polymerase	β-subunit of bacterial RNA-polymerase	32.8	These autoantibodies in PBC, suggest bacterial triggers of PBC	[180]
46	Anti-EPO	Eosinophil peroxidase	52.5	PBC patients with positive anti-EPO antibodies have less peripheral eosinophils	[181]
47	Anti-p53	Nuclear protein of 53 kDa that regulates cell proliferation and apoptosis	8.0	Anti-p53 autoantibodies are commonly seen in malignancies and organ-specific autoimmune diseases such as type 1 diabetes, thyroid diseases, PBC and AIH	[182]
48	Anti-acetylcholine receptor	Nicotinic acetylcholine receptor	58.8-74.0	Anti-acetylcholine receptor antibodies are primarily associated with myasthenia gravis, though PBC patients with positive anti-acetylcholine receptor antibodies do not have clinical symptoms of myasthenia	[169,183,184]
49	Anti-CA Ⅱ	Carbonic anhydrase Ⅱ	18-31	Anti-CA Ⅱ antibody is likely a nonspecific marker of autoimmunity. It has been detected in a variety of autoimmune diseases, including Graves' disease, type 1 diabetes, SS, SLE, AIH and PBC. In cases of PBC, no significant correlation has been found between anti-CA Ⅱ antibody and AMA	[185-189]
50	Anti-α enolase	α-enolase	28.6	Anti-α-enolase antibodies present in a variety of inflammatory and autoimmune disorders, such as SLE, IBD, RA and AIH, and are not likely to be specific markers for any disease. They might be involved in destruction of biliary epithelium and are associated with hepatic failure	[190-195]
51	Anti-HSP	Heat shock proteins	45.7	Enhanced biliary expression of heat shock protein is found in PBC. Anti-HSPs are common in PBC, and are related to titers of AMA. They might cross-react with the main mitochondrial antigens in PBC	[196-199]
52	Anti-FKBP12	FK506 binding protein 12	44.4	The significance of anti-FKBP12 antibodies in PBC is unclear	[200]

SLA: Soluble liver antigen; PBC: Primary biliary cirrhosis; AMA: Antimitochondrial antibodies; SLE: Systemic lupus erythematosus; SSc: Systemic sclerosis; ANA: Antinuclear antibodies.

disease, defined as the presence of cirrhosis or its complications. In addition, it has been reported that anti-p62-positive patients have higher levels of serum bilirubin and more marked inflammatory infiltrates on liver biopsy[87].

Antinuclear envelope antibodies (Lamin and Lamin B receptor)

The nuclear envelope is a bilayered membranous structure that can be divided into five distinct components: the inner nuclear membrane, having a distinct set of integral membrane proteins; the outer nuclear membrane; a perinuclear space, which is continuous with the lumen of the endoplasmic reticulum; the pore domains, regions where the inner nuclear membrane and outer nuclear membrane come together and fuse; and an underlying nuclear lamina, containing the nuclear lamins[103]. A smooth membrane fluorescence pattern is characteristic of the presence of antibodies to nuclear lamins in IIF using sera from PBC patients. Three subtypes of anti-lamin antibodies have been described: anti-lamin A, B and C[102,104-106]. Anti-lamin antibodies do not seem to be disease-specific as they are found in patients with several different autoimmune disorders, such as SLE, chronic fatigue syndrome, and PBC[107-110]. Anti-lamin A, B and C antibodies are detected with frequencies of 6%-8% in sera from patients with PBC. The usual scenario is to find anti-lamin A and C together, and less frequently either anti-lamin B alone or all three in the same patient[111].

Lamin B receptor (LBR) is a protein integral to the inner nuclear membrane with a nucleoplasmic, amino-terminal domain of 208 amino acids, followed by a carboxyl-terminal domain with eight putative transmembrane segments. Anti-LBR antibodies from PBC patients recognize the nucleoplasmic, amino-terminal domain but not the carboxyl-terminal domain. Anti-LBR antibodies appear to be highly specific for PBC, but their clinical significance is unclear. The prevalence of anti-LBR antibodies in PBC is approximately 2%-6%[76,102,112,113].

Anti-centromere antibodies

Anti-centromere antibodies (ACA) are important diagnostic markers of systemic sclerosis (SSc), found in about 25% of these patients[114]. In patients with CREST syndrome or limited cutaneous SSc, the positivity rises to 50%-90%. ACA in SSc are usually associated with a good prognosis, though they are not specific for SSc. ACA can be detected in patients with other rheumatic diseases including pSS, SLE and PBC (about 30%)[115-120]. It is of interest to note that several subtypes of ACA have been identified, including anti-CENP-A, anti-CENP-B, anti-CENP-C and anti-CENP-O antibodies[121]. Research during the past several years has found that prevalence of the ACA subtypes

differs among various autoimmune diseases[122]. Recent studies have demonstrated that ACA positivity in patients with PBC is of significant predictive value for progression to portal hypertension[123,124].

OTHER AUTOANTIBODIES DETECTED IN PBC

Although extensive research has focused on AMA, it is of interest to note that, to date, more than sixty different autoantibodies have been found in PBC patients. Some target at nuclear or cytoplasmic molecules and cell membranes, while others react with lipid components. Some, like AMA, occur frequently and almost universally in PBC, while others, like anti-lamin and anti-LBR, are present in only a few patients. It should be noted that among these autoantibodies, some are not specific for any disease, and some are thought to be more closely related to other autoimmune diseases, such as anti-CCP which is relatively specific for rheumatoid arthritis[125,126]. Prevalence and properties of these autoantibodies in PBC are summarized in Table 1.

CONCLUSION

The presence of serum autoantibodies is characteristic of PBC, and is useful in the clinical diagnostic process in combination with histology and imaging studies. Numerous autoantibodies are found in sera from patients with PBC. This suggests that the development of PBC is a multi-factorial process. With growing numbers of clinical studies of autoimmune diseases and extensive application of more sensitive testing methods for antibodies, it has gradually been realized that the association between an individual autoantibody and autoimmune disease is not as specific as previously thought. AMA is very sensitive and anti-gp210 and anti-sp100 are highly specific for PBC. Other antibodies found in PBC, such as ACA, ASCA, ANCA and anti-sm, could also be found in other autoimmune diseases[131,161,162,168]. Although some autoantibodies are believed to be associated with the pathogenesis of PBC, these associations are likely to be extremely complicated and surely exert complex effects in many different ways. It is hard to understand these delicate associations based on our current knowledge of PBC, and further advanced studies are required to elucidate the pathogenesis of this autoimmune disease.

REFERENCES

1 **Kaplan MM**, Gershwin ME. Primary biliary cirrhosis. *N Engl J Med* 2005; **353**: 1261-1273
2 **Prince M**, Chetwynd A, Newman W, Metcalf JV, James OF. Survival and symptom progression in a geographically based cohort of patients with primary biliary cirrhosis: follow-up for up to 28 years. *Gastroenterology* 2002; **123**: 1044-1051
3 **Kim WR**, Lindor KD, Locke GR 3rd, Therneau TM, Homburger HA, Batts KP, Yawn BP, Petz JL, Melton LJ 3rd, Dickson ER. Epidemiology and natural history of primary biliary cirrhosis in a US community. *Gastroenterology* 2000; **119**: 1631-1636
4 **Selmi C**, Invernizzi P, Zuin M, Podda M, Gershwin ME. Genetics and geoepidemiology of primary biliary cirrhosis: following the footprints to disease etiology. *Semin Liver Dis* 2005; **25**: 265-280
5 **Prince MI**, James OF. The epidemiology of primary biliary cirrhosis. *Clin Liver Dis* 2003; **7**: 795-819
6 **Selmi C**, Mayo MJ, Bach N, Ishibashi H, Invernizzi P, Gish RG, Gordon SC, Wright HI, Zweiban B, Podda M, Gershwin ME. Primary biliary cirrhosis in monozygotic and dizygotic twins: genetics, epigenetics, and environment. *Gastroenterology* 2004; **127**: 485-492
7 **Lazaridis KN**, Juran BD, Boe GM, Slusser JP, de Andrade M, Homburger HA, Ghosh K, Dickson ER, Lindor KD, Petersen GM. Increased prevalence of antimitochondrial antibodies in first-degree relatives of patients with primary biliary cirrhosis. *Hepatology* 2007; **46**: 785-792
8 **Allina J**, Hu B, Sullivan DM, Fiel MI, Thung SN, Bronk SF, Huebert RC, van de Water J, LaRusso NF, Gershwin ME, Gores GJ, Odin JA. T cell targeting and phagocytosis of apoptotic biliary epithelial cells in primary biliary cirrhosis. *J Autoimmun* 2006; **27**: 232-241
9 **He XS**, Ansari AA, Ridgway WM, Coppel RL, Gershwin ME. New insights to the immunopathology and autoimmune responses in primary biliary cirrhosis. *Cell Immunol* 2006; **239**: 1-13
10 **Kita H**. Autoreactive CD8-specific T-cell response in primary biliary cirrhosis. *Hepatol Res* 2007; **37** Suppl 3: S402-S405
11 **Ichiki Y**, Shimoda S, Hara H, Shigematsu H, Nakamura M, Hayashida K, Ishibashi H, Niho Y. Analysis of T-cell receptor beta of the T-cell clones reactive to the human PDC-E2 163-176 peptide in the context of HLA-DR53 in patients with primary biliary cirrhosis. *Hepatology* 1997; **26**: 728-733
12 **Moteki S**, Leung PS, Dickson ER, Van Thiel DH, Galperin C, Buch T, Alarcon-Segovia D, Kershenobich D, Kawano K, Coppel RL. Epitope mapping and reactivity of autoantibodies to the E2 component of 2-oxoglutarate dehydrogenase complex in primary biliary cirrhosis using recombinant 2-oxoglutarate dehydrogenase complex. *Hepatology* 1996; **23**: 436-444
13 **Meda F**, Zuin M, Invernizzi P, Vergani D, Selmi C. Serum autoantibodies: a road map for the clinical hepatologist. *Autoimmunity* 2008; **41**: 27-34
14 **Abe M**, Onji M. Natural history of primary biliary cirrhosis. *Hepatol Res* 2008; **38**: 639-645
15 **Oertelt S**, Rieger R, Selmi C, Invernizzi P, Ansari AA, Coppel RL, Podda M, Leung PS, Gershwin ME. A sensitive bead assay for antimitochondrial antibodies: Chipping away at AMA-negative primary biliary cirrhosis. *Hepatology* 2007; **45**: 659-665
16 **Neuberger J**, Thomson R. PBC and AMA--what is the connection? *Hepatology* 1999; **29**: 271-276
17 **Gajdusek DC**. An autoimmune reaction against human tissue antigens in certain acute and chronic diseases. I. Serological investigations. *AMA Arch Intern Med* 1958; **101**: 9-29
18 **Walker JG**, Doniach D, Roitt IM, Sherlock S. Serological tests in diagnosis of primary biliary cirrhosis. *Lancet* 1965; **1**: 827-831
19 **Lindenborn-Fotinos J**, Baum H, Berg PA. Mitochondrial antibodies in primary biliary cirrhosis: species and nonspecies specific determinants of M2 antigen. *Hepatology* 1985; **5**: 763-769
20 **Gershwin ME**, Mackay IR, Sturgess A, Coppel RL. Identification and specificity of a cDNA encoding the 70 kd mitochondrial antigen recognized in primary biliary cirrhosis. *J Immunol* 1987; **138**: 3525-3531
21 **Reynoso-Paz S**, Leung PS, Van De Water J, Tanaka A, Munoz S, Bass N, Lindor K, Donald PJ, Coppel RL, Ansari AA, Gershwin ME. Evidence for a locally driven mucosal response and the presence of mitochondrial antigens in saliva in primary biliary cirrhosis. *Hepatology* 2000; **31**: 24-29

22 **Nishio A**, Van de Water J, Leung PS, Joplin R, Neuberger JM, Lake J, Björkland A, Tötterman TH, Peters M, Worman HJ, Ansari AA, Coppel RL, Gershwin ME. Comparative studies of antimitochondrial autoantibodies in sera and bile in primary biliary cirrhosis. *Hepatology* 1997; **25**: 1085-1089
23 **Tanaka A**, Nezu S, Uegaki S, Mikami M, Okuyama S, Kawamura N, Aiso M, Gershwin ME, Takahashi S, Selmi C, Takikawa H. The clinical significance of IgA antimitochondrial antibodies in sera and saliva in primary biliary cirrhosis. *Ann N Y Acad Sci* 2007; **1107**: 259-270
24 **Berg PA**, Wiedmann KH, Sayers T, Klöppel G, Lindner H. Serological classification of chronic cholestatic liver disease by the use of two different types of antimitochondrial antibodies. *Lancet* 1980; **2**: 1329-1332
25 **Berg CP**, Stein GM, Klein R, Pascu M, Berg T, Kammer W, Priemer M, Nordheim A, Schulze-Osthoff K, Gregor M, Wesselborg S, Berg PA. Demonstration of PDC-E1 subunits as major antigens in the complement-fixing fraction M4 and re-evaluation of PDC-E1-specific antibodies in PBC patients. *Liver Int* 2006; **26**: 846-855
26 **Preuss B**, Berg C, Altenberend F, Gregor M, Stevanovic S, Klein R. Demonstration of autoantibodies to recombinant human sulphite oxidase in patients with chronic liver disorders and analysis of their clinical relevance. *Clin Exp Immunol* 2007; **150**: 312-321
27 **Weber P**, Brenner J, Stechemesser E, Klein R, Weckenmann U, Klöppel G, Kirchhof M, Fintelmann V, Berg PA. Characterization and clinical relevance of a new complement-fixing antibody--anti-M8--in patients with primary biliary cirrhosis. *Hepatology* 1986; **6**: 553-559
28 **Klein R**, Berg PA. Characterization of a new mitochondrial antigen-antibody system (M9/anti-M9) in patients with anti-M2 positive and anti-M2 negative primary biliary cirrhosis. *Clin Exp Immunol* 1988; **74**: 68-74
29 **Berg PA**, Klein R. Mitochondrial antigen/antibody systems in primary biliary cirrhosis: revisited. *Liver* 1995; **15**: 281-292
30 **Klein R**, Pointner H, Zilly W, Glässner-Bittner B, Breuer N, Garbe W, Fintelmann V, Kalk JF, Müting D, Fischer R, Tittor W, Pausch J, Maier KP, Berg PA. Antimitochondrial antibody profiles in primary biliary cirrhosis distinguish at early stages between a benign and a progressive course: a prospective study on 200 patients followed for 10 years. *Liver* 1997; **17**: 119-128
31 **Klein R**, Klöppel G, Garbe W, Fintelmann V, Berg PA. Antimitochondrial antibody profiles determined at early stages of primary biliary cirrhosis differentiate between a benign and a progressive course of the disease. A retrospective analysis of 76 patients over 6-18 years. *J Hepatol* 1991; **12**: 21-27
32 **Vleggaar FP**, van Buuren HR. No prognostic significance of antimitochondrial antibody profile testing in primary biliary cirrhosis. *Hepatogastroenterology* 2004; **51**: 937-940
33 **Chung L**, Utz PJ. Antibodies in scleroderma: direct pathogenicity and phenotypic associations. *Curr Rheumatol Rep* 2004; **6**: 156-163
34 **O'Brien C**, Joshi S, Feld JJ, Guindi M, Dienes HP, Heathcote EJ. Long-term follow-up of antimitochondrial antibody-positive autoimmune hepatitis. *Hepatology* 2008; **48**: 550-556
35 **Nardi N**, Brito-Zerón P, Ramos-Casals M, Aguiló S, Cervera R, Ingelmo M, Font J. Circulating auto-antibodies against nuclear and non-nuclear antigens in primary Sjögren's syndrome: prevalence and clinical significance in 335 patients. *Clin Rheumatol* 2006; **25**: 341-346
36 **Ramos-Casals M**, Pares A, Jara LJ, Solans R, Viñas O, Vázquez P, Sánchez-Tapias JM, Rodés J, Font J. Antimitochondrial antibodies in patients with chronic hepatitis C virus infection: description of 18 cases and review of the literature. *J Viral Hepat* 2005; **12**: 648-654
37 **Montano-Loza AJ**, Carpenter HA, Czaja AJ. Frequency, behavior, and prognostic implications of antimitochondrial antibodies in type 1 autoimmune hepatitis. *J Clin Gastroenterol* 2008; **42**: 1047-1053
38 **Klein R**, Wiebel M, Engelhart S, Berg PA. Sera from patients with tuberculosis recognize the M2a-epitope (E2-subunit of pyruvate dehydrogenase) specific for primary biliary cirrhosis. *Clin Exp Immunol* 1993; **92**: 308-316
39 **Shimoda S**, Van de Water J, Ansari A, Nakamura M, Ishibashi H, Coppel RL, Lake J, Keeffe EB, Roche TE, Gershwin ME. Identification and precursor frequency analysis of a common T cell epitope motif in mitochondrial autoantigens in primary biliary cirrhosis. *J Clin Invest* 1998; **102**: 1831-1840
40 **Kamihira T**, Shimoda S, Harada K, Kawano A, Handa M, Baba E, Tsuneyama K, Nakamura M, Ishibashi H, Nakanuma Y, Gershwin ME, Harada M. Distinct costimulation dependent and independent autoreactive T-cell clones in primary biliary cirrhosis. *Gastroenterology* 2003; **125**: 1379-1387
41 **Kita H**, Ansari AA, He XS, Lian ZX, Van de Water J, Coppel RL, Luketic V, Kaplan M, Inamori H, Isoda N, Sugano K, Imawari M, Gershwin ME. Proteasome is required for class I-restricted presentation by Fcgamma receptor-mediated endocytosis in primary biliary cirrhosis. *J Autoimmun* 2003; **21**: 175-182
42 **Kita H**, Matsumura S, He XS, Ansari AA, Lian ZX, Van de Water J, Coppel RL, Kaplan MM, Gershwin ME. Quantitative and functional analysis of PDC-E2-specific autoreactive cytotoxic T lymphocytes in primary biliary cirrhosis. *J Clin Invest* 2002; **109**: 1231-1240
43 **Tanimoto H**, Shimoda S, Nakamura M, Ishibashi H, Kawano A, Kamihira T, Matsushita S, Gershwin ME, Harada M. Promiscuous T cells selected by Escherichia coli: OGDC-E2 in primary biliary cirrhosis. *J Autoimmun* 2003; **20**: 255-263
44 **Kita H**, Lian ZX, Van de Water J, He XS, Matsumura S, Kaplan M, Luketic V, Coppel RL, Ansari AA, Gershwin ME. Identification of HLA-A2-restricted CD8(+) cytotoxic T cell responses in primary biliary cirrhosis: T cell activation is augmented by immune complexes cross-presented by dendritic cells. *J Exp Med* 2002; **195**: 113-123
45 **Poupon RE**, Balkau B, Eschwège E, Poupon R. A multicenter, controlled trial of ursodiol for the treatment of primary biliary cirrhosis. UDCA-PBC Study Group. *N Engl J Med* 1991; **324**: 1548-1554
46 **Benson GD**, Kikuchi K, Miyakawa H, Tanaka A, Watnik MR, Gershwin ME. Serial analysis of antimitochondrial antibody in patients with primary biliary cirrhosis. *Clin Dev Immunol* 2004; **11**: 129-133
47 **Luettig B**, Boeker KH, Schoessler W, Will H, Loges S, Schmidt E, Worman HJ, Gershwin ME, Manns MP. The antinuclear autoantibodies Sp100 and gp210 persist after orthotopic liver transplantation in patients with primary biliary cirrhosis. *J Hepatol* 1998; **28**: 824-828
48 **Matsumura S**, Van De Water J, Leung P, Odin JA, Yamamoto K, Gores GJ, Mostov K, Ansari AA, Coppel RL, Shiratori Y, Gershwin ME. Caspase induction by IgA antimitochondrial antibody: IgA-mediated biliary injury in primary biliary cirrhosis. *Hepatology* 2004; **39**: 1415-1422
49 **Selmi C**, Zuin M, Gershwin ME. The unfinished business of primary biliary cirrhosis. *J Hepatol* 2008; **49**: 451-460
50 **Rigopoulou EI**, Davies ET, Bogdanos DP, Liaskos C, Mytilinaiou M, Koukoulis GK, Dalekos GN, Vergani D. Antimitochondrial antibodies of immunoglobulin G3 subclass are associated with a more severe disease course in primary biliary cirrhosis. *Liver Int* 2007; **27**: 1226-1231
51 **Lleo A**, Selmi C, Invernizzi P, Podda M, Gershwin ME. The consequences of apoptosis in autoimmunity. *J Autoimmun* 2008; **31**: 257-262
52 **Lleo A**, Invernizzi P, Selmi C, Coppel RL, Alpini G, Podda M, Mackay IR, Gershwin ME. Autophagy: highlighting a novel player in the autoimmunity scenario. *J Autoimmun* 2007; **29**: 61-68
53 **Leung PS**, Rossaro L, Davis PA, Park O, Tanaka A, Kikuchi K, Miyakawa H, Norman GL, Lee W, Gershwin ME. Antimi-

tochondrial antibodies in acute liver failure: implications for primary biliary cirrhosis. *Hepatology* 2007; **46**: 1436-1442
54 **Bogdanos DP**, Choudhuri K, Vergani D. Molecular mimicry and autoimmune liver disease: virtuous intentions, malign consequences. *Liver* 2001; **21**: 225-232
55 **Sutton I**, Neuberger J. Primary biliary cirrhosis: seeking the silent partner of autoimmunity. *Gut* 2002; **50**: 743-746
56 **Long SA**, Quan C, Van de Water J, Nantz MH, Kurth MJ, Barsky D, Colvin ME, Lam KS, Coppel RL, Ansari A, Gershwin ME. Immunoreactivity of organic mimeotopes of the E2 component of pyruvate dehydrogenase: connecting xenobiotics with primary biliary cirrhosis. *J Immunol* 2001; **167**: 2956-2963
57 **Odin JA**, Huebert RC, Casciola-Rosen L, LaRusso NF, Rosen A. Bcl-2-dependent oxidation of pyruvate dehydrogenase-E2, a primary biliary cirrhosis autoantigen, during apoptosis. *J Clin Invest* 2001; **108**: 223-232
58 **Allina J**, Stanca CM, Garber J, Hu B, Sautes-Fridman C, Bach N, Odin JA. Anti-CD16 autoantibodies and delayed phagocytosis of apoptotic cells in primary biliary cirrhosis. *J Autoimmun* 2008; **30**: 238-245
59 **Berg CP**, Stein GM, Keppeler H, Gregor M, Wesselborg S, Lauber K. Apoptosis-associated antigens recognized by autoantibodies in patients with the autoimmune liver disease primary biliary cirrhosis. *Apoptosis* 2008; **13**: 63-75
60 **Muratori P**, Muratori L, Gershwin ME, Czaja AJ, Pappas G, MacCariello S, Granito A, Cassani F, Loria P, Lenzi M, Bianchi FB. 'True' antimitochondrial antibody-negative primary biliary cirrhosis, low sensitivity of the routine assays, or both? *Clin Exp Immunol* 2004; **135**: 154-158
61 **Hirschfield GM**, Heathcote EJ. Antimitochondrial antibody-negative primary biliary cirrhosis. *Clin Liver Dis* 2008; **12**: 323-331; viii-ix
62 **Liu B**, Shi XH, Zhang FC, Zhang W, Gao LX. Antimitochondrial antibody-negative primary biliary cirrhosis: a subset of primary biliary cirrhosis. *Liver Int* 2008; **28**: 233-239
63 **Zhang FK**, Jia JD, Wang BE. Clinical evaluation of serum antimitochondrial antibody-negative primary biliary cirrhosis. *Hepatobiliary Pancreat Dis Int* 2004; **3**: 288-291
64 **Dähnrich C**, Pares A, Caballeria L, Rosemann A, Schlumberger W, Probst C, Mytilinaiou M, Bogdanos D, Vergani D, Stöcker W, Komorowski L. New ELISA for detecting primary biliary cirrhosis-specific antimitochondrial antibodies. *Clin Chem* 2009; **55**: 978-985
65 **Mendes F**, Lindor KD. Antimitochondrial antibody-negative primary biliary cirrhosis. *Gastroenterol Clin North Am* 2008; **37**: 479-484, viii
66 **Szostecki C**, Krippner H, Penner E, Bautz FA. Autoimmune sera recognize a 100 kD nuclear protein antigen (sp-100). *Clin Exp Immunol* 1987; **68**: 108-116
67 **Fusconi M**, Cassani F, Govoni M, Caselli A, Farabegoli F, Lenzi M, Ballardini G, Zauli D, Bianchi FB. Anti-nuclear antibodies of primary biliary cirrhosis recognize 78-92-kD and 96-100-kD proteins of nuclear bodies. *Clin Exp Immunol* 1991; **83**: 291-297
68 **Morriswood B**, Ryzhakov G, Puri C, Arden SD, Roberts R, Dendrou C, Kendrick-Jones J, Buss F. T6BP and NDP52 are myosin VI binding partners with potential roles in cytokine signalling and cell adhesion. *J Cell Sci* 2007; **120**: 2574-2585
69 **Thurston TL**, Ryzhakov G, Bloor S, von Muhlinen N, Randow F. The TBK1 adaptor and autophagy receptor NDP52 restricts the proliferation of ubiquitin-coated bacteria. *Nat Immunol* 2009; **10**: 1215-1221
70 **Bernstein RM**, Neuberger JM, Bunn CC, Callender ME, Hughes GR, Williams R. Diversity of autoantibodies in primary biliary cirrhosis and chronic active hepatitis. *Clin Exp Immunol* 1984; **55**: 553-560
71 **Sternsdorf T**, Guldner HH, Szostecki C, Grötzinger T, Will H. Two nuclear dot-associated proteins, PML and Sp100, are often co-autoimmunogenic in patients with primary biliary cirrhosis. *Scand J Immunol* 1995; **42**: 257-268
72 **Szostecki C**, Will H, Netter HJ, Guldner HH. Autoantibodies to the nuclear Sp100 protein in primary biliary cirrhosis and associated diseases: epitope specificity and immunoglobulin class distribution. *Scand J Immunol* 1992; **36**: 555-564
73 **Pawlotsky JM**, Andre C, Metreau JM, Beaugrand M, Zafrani ES, Dhumeaux D. Multiple nuclear dots antinuclear antibodies are not specific for primary biliary cirrhosis. *Hepatology* 1992; **16**: 127-131
74 **Wichmann I**, Montes-Cano MA, Respaldiza N, Alvarez A, Walter K, Franco E, Sanchez-Roman J, Núñez-Roldán A. Clinical significance of anti-multiple nuclear dots/Sp100 autoantibodies. *Scand J Gastroenterol* 2003; **38**: 996-999
75 **Worman HJ**, Courvalin JC. Antinuclear antibodies specific for primary biliary cirrhosis. *Autoimmun Rev* 2003; **2**: 211-217
76 **Muratori P**, Muratori L, Ferrari R, Cassani F, Bianchi G, Lenzi M, Rodrigo L, Linares A, Fuentes D, Bianchi FB. Characterization and clinical impact of antinuclear antibodies in primary biliary cirrhosis. *Am J Gastroenterol* 2003; **98**: 431-437
77 **Manuel Lucena J**, Montes Cano M, Luis Caro J, Respaldiza N, Alvarez A, Sánchez-Román J, Núñez-Roldán A, Wichmann I. Comparison of two ELISA assays for anti-Sp100 determination. *Ann N Y Acad Sci* 2007; **1109**: 203-211
78 **Milkiewicz P**, Buwaneswaran H, Coltescu C, Shums Z, Norman GL, Heathcote EJ. Value of autoantibody analysis in the differential diagnosis of chronic cholestatic liver disease. *Clin Gastroenterol Hepatol* 2009; **7**: 1355-1360
79 **Bogdanos DP**, Baum H, Butler P, Rigopoulou EI, Davies ET, Ma Y, Burroughs AK, Vergani D. Association between the primary biliary cirrhosis specific anti-sp100 antibodies and recurrent urinary tract infection. *Dig Liver Dis* 2003; **35**: 801-805
80 **Kakizuka A**, Miller WH Jr, Umesono K, Warrell RP Jr, Frankel SR, Murty VV, Dmitrovsky E, Evans RM. Chromosomal translocation t(15;17) in human acute promyelocytic leukemia fuses RAR alpha with a novel putative transcription factor, PML. *Cell* 1991; **66**: 663-674
81 **Borden KL**. Pondering the promyelocytic leukemia protein (PML) puzzle: possible functions for PML nuclear bodies. *Mol Cell Biol* 2002; **22**: 5259-5269
82 **Bloch DB**, Nakajima A, Gulick T, Chiche JD, Orth D, de La Monte SM, Bloch KD. Sp110 localizes to the PML-Sp100 nuclear body and may function as a nuclear hormone receptor transcriptional coactivator. *Mol Cell Biol* 2000; **20**: 6138-6146
83 **Züchner D**, Sternsdorf T, Szostecki C, Heathcote EJ, Cauch-Dudek K, Will H. Prevalence, kinetics, and therapeutic modulation of autoantibodies against Sp100 and promyelocytic leukemia protein in a large cohort of patients with primary biliary cirrhosis. *Hepatology* 1997; **26**: 1123-1130
84 **Szostecki C**, Guldner HH, Will H. Autoantibodies against "nuclear dots" in primary biliary cirrhosis. *Semin Liver Dis* 1997; **17**: 71-78
85 **Granito A**, Yang WH, Muratori L, Lim MJ, Nakajima A, Ferri S, Pappas G, Quarneti C, Bianchi FB, Bloch DB, Muratori P. PML nuclear body component Sp140 is a novel autoantigen in primary biliary cirrhosis. *Am J Gastroenterol* 2010; **105**: 125-131
86 **Ruffatti A**, Arslan P, Floreani A, De Silvestro G, Calligaro A, Naccarato R, Todesco S. Nuclear membrane-staining antinuclear antibody in patients with primary biliary cirrhosis. *J Clin Immunol* 1985; **5**: 357-361
87 **Invernizzi P**, Podda M, Battezzati PM, Crosignani A, Zuin M, Hitchman E, Maggioni M, Meroni PL, Penner E, Wesierska-Gadek J. Autoantibodies against nuclear pore complexes are associated with more active and severe liver disease in primary biliary cirrhosis. *J Hepatol* 2001; **34**: 366-372
88 **Rigopoulou EI**, Davies ET, Pares A, Zachou K, Liaskos C, Bogdanos DP, Rodes J, Dalekos GN, Vergani D. Prevalence and clinical significance of isotype specific antinuclear antibodies in primary biliary cirrhosis. *Gut* 2005; **54**: 528-532
89 **Muratori P**, Granito A, Ferri S, Pappas G, Volta U, Menichel-

la R, Bianchi FB, Lenzi M, Muratori L. Multiple nuclear dots and rim-like/membranous IgG isotypes in primary biliary cirrhosis. *Autoimmunity* 2009; **42**: 224-227
90 **Lassoued K**, Brenard R, Degos F, Courvalin JC, Andre C, Danon F, Brouet JC, Zine-el-Abidine Y, Degott C, Zafrani S. Antinuclear antibodies directed to a 200-kilodalton polypeptide of the nuclear envelope in primary biliary cirrhosis. A clinical and immunological study of a series of 150 patients with primary biliary cirrhosis. *Gastroenterology* 1990; **99**: 181-186
91 **Courvalin JC**, Lassoued K, Bartnik E, Blobel G, Wozniak RW. The 210-kD nuclear envelope polypeptide recognized by human autoantibodies in primary biliary cirrhosis is the major glycoprotein of the nuclear pore. *J Clin Invest* 1990; **86**: 279-285
92 **Worman HJ**. Nuclear envelope protein autoantigens in primary biliary cirrhosis. *Hepatol Res* 2007; **37** Suppl 3: S406-S411
93 **Gao L**, Tian X, Liu B, Zhang F. The value of antinuclear antibodies in primary biliary cirrhosis. *Clin Exp Med* 2008; **8**: 9-15
94 **Bauer A**, Habior A. Measurement of gp210 autoantibodies in sera of patients with primary biliary cirrhosis. *J Clin Lab Anal* 2007; **21**: 227-231
95 **Nakamura M**, Shimizu-Yoshida Y, Takii Y, Komori A, Yokoyama T, Ueki T, Daikoku M, Yano K, Matsumoto T, Migita K, Yatsuhashi H, Ito M, Masaki N, Adachi H, Watanabe Y, Nakamura Y, Saoshiro T, Sodeyama T, Koga M, Shimoda S, Ishibashi H. Antibody titer to gp210-C terminal peptide as a clinical parameter for monitoring primary biliary cirrhosis. *J Hepatol* 2005; **42**: 386-392
96 **Nakamura M**, Komori A, Ito M, Kondo H, Aiba Y, Migita K, Nagaoka S, Ohata K, Yano K, Abiru S, Daikoku M, Yatsuhashi H, Shimoda S, Ishibashi H. Predictive role of anti-gp210 and anticentromere antibodies in long-term outcome of primary biliary cirrhosis. *Hepatol Res* 2007; **37** Suppl 3: S412-S419
97 **Nakamura M**, Takii Y, Ito M, Komori A, Yokoyama T, Shimizu-Yoshida Y, Koyabu M, Matsuyama M, Mori T, Kamihira T, Daikoku M, Migita K, Yatsuhashi H, Nozaki N, Shimoda S, Ishibashi H. Increased expression of nuclear envelope gp210 antigen in small bile ducts in primary biliary cirrhosis. *J Autoimmun* 2006; **26**: 138-145
98 **Davis LI**, Blobel G. Nuclear pore complex contains a family of glycoproteins that includes p62: glycosylation through a previously unidentified cellular pathway. *Proc Natl Acad Sci USA* 1987; **84**: 7552-7556
99 **Wesierska-Gadek J**, Hohenuer H, Hitchman E, Penner E. Autoantibodies against nucleoporin p62 constitute a novel marker of primary biliary cirrhosis. *Gastroenterology* 1996; **110**: 840-847
100 **Invernizzi P**, Selmi C, Ranftler C, Podda M, Wesierska-Gadek J. Antinuclear antibodies in primary biliary cirrhosis. *Semin Liver Dis* 2005; **25**: 298-310
101 **Wesierska-Gadek J**, Klima A, Komina O, Ranftler C, Invernizzi P, Penner E. Characterization of autoantibodies against components of the nuclear pore complexes: high frequency of anti-p62 nucleoporin antibodies. *Ann N Y Acad Sci* 2007; **1109**: 519-530
102 **Miyachi K**, Hankins RW, Matsushima H, Kikuchi F, Inomata T, Horigome T, Shibata M, Onozuka Y, Ueno Y, Hashimoto E, Hayashi N, Shibuya A, Amaki S, Miyakawa H. Profile and clinical significance of anti-nuclear envelope antibodies found in patients with primary biliary cirrhosis: a multicenter study. *J Autoimmun* 2003; **20**: 247-254
103 **Salina D**, Bodoor K, Enarson P, Raharjo WH, Burke B. Nuclear envelope dynamics. *Biochem Cell Biol* 2001; **79**: 533-542
104 **Fisher DZ**, Chaudhary N, Blobel G. cDNA sequencing of nuclear lamins A and C reveals primary and secondary structural homology to intermediate filament proteins. *Proc Natl Acad Sci USA* 1986; **83**: 6450-6454
105 **Krohne G**, Wolin SL, McKeon FD, Franke WW, Kirschner MW. Nuclear lamin LI of Xenopus laevis: cDNA cloning, amino acid sequence and binding specificity of a member of the lamin B subfamily. *EMBO J* 1987; **6**: 3801-3808
106 **Wesierska-Gadek J**, Penner E, Hitchman E, Sauermann G. Antibodies to nuclear lamins in autoimmune liver disease. *Clin Immunol Immunopathol* 1988; **49**: 107-115
107 **Senécal JL**, Ichiki S, Girard D, Raymond Y. Autoantibodies to nuclear lamins and to intermediate filament proteins: natural, pathologic or pathogenic? *J Rheumatol* 1993; **20**: 211-219
108 **Konstantinov K**, von Mikecz A, Buchwald D, Jones J, Gerace L, Tan EM. Autoantibodies to nuclear envelope antigens in chronic fatigue syndrome. *J Clin Invest* 1996; **98**: 1888-1896
109 **Dieudé M**, Senécal JL, Rauch J, Hanly JG, Fortin P, Brassard N, Raymond Y. Association of autoantibodies to nuclear lamin B1 with thromboprotection in systemic lupus erythematosus: lack of evidence for a direct role of lamin B1 in apoptotic blebs. *Arthritis Rheum* 2002; **46**: 2695-2707
110 **Coppo P**, Clauvel JP, Bengoufa D, Fuentes V, Gouilleux-Gruart V, Courvalin JC, Lassoued K. Autoimmune cytopenias associated with autoantibodies to nuclear envelope polypeptides. *Am J Hematol* 2004; **77**: 241-249
111 **Nesher G**, Margalit R, Ashkenazi YJ. Anti-nuclear envelope antibodies: Clinical associations. *Semin Arthritis Rheum* 2001; **30**: 313-320
112 **Lin F**, Noyer CM, Ye Q, Courvalin JC, Worman HJ. Autoantibodies from patients with primary biliary cirrhosis recognize a region within the nucleoplasmic domain of inner nuclear membrane protein LBR. *Hepatology* 1996; **23**: 57-61
113 **Courvalin JC**, Lassoued K, Worman HJ, Blobel G. Identification and characterization of autoantibodies against the nuclear envelope lamin B receptor from patients with primary biliary cirrhosis. *J Exp Med* 1990; **172**: 961-967
114 **Vázquez-Abad D**, Rothfield NF. Autoantibodies in systemic sclerosis. *Int Rev Immunol* 1995; **12**: 145-157
115 **Salliot C**, Gottenberg JE, Bengoufa D, Desmoulins F, Miceli-Richard C, Mariette X. Anticentromere antibodies identify patients with Sjögren's syndrome and autoimmune overlap syndrome. *J Rheumatol* 2007; **34**: 2253-2258
116 **Yan SM**, Zeng XF, Zhao Y, Dong Y. [A clinical analysis of primary Sjögren's syndrome with anticentromere antibodies] *Zhonghua Neike Zazhi* 2008; **47**: 296-299
117 **Vlachoyiannopoulos PG**, Drosos AA, Wiik A, Moutsopoulos HM. Patients with anticentromere antibodies, clinical features, diagnoses and evolution. *Br J Rheumatol* 1993; **32**: 297-301
118 **Nakano M**, Ohuchi Y, Hasegawa H, Kuroda T, Ito S, Gejyo F. Clinical significance of anticentromere antibodies in patients with systemic lupus erythematosus. *J Rheumatol* 2000; **27**: 1403-1407
119 **Chan HL**, Lee YS, Hong HS, Kuo TT. Anticentromere antibodies (ACA): clinical distribution and disease specificity. *Clin Exp Dermatol* 1994; **19**: 298-302
120 **Parveen S**, Morshed SA, Nishioka M. High prevalence of antibodies to recombinant CENP-B in primary biliary cirrhosis: nuclear immunofluorescence patterns and ELISA reactivities. *J Gastroenterol Hepatol* 1995; **10**: 438-445
121 **Akbarali Y**, Matousek-Ronck J, Hunt L, Staudt L, Reichlin M, Guthridge JM, James JA. Fine specificity mapping of autoantigens targeted by anti-centromere autoantibodies. *J Autoimmun* 2006; **27**: 272-280
122 **Russo K**, Hoch S, Dima C, Varga J, Teodorescu M. Circulating anticentromere CENP-A and CENP-B antibodies in patients with diffuse and limited systemic sclerosis, systemic lupus erythematosus, and rheumatoid arthritis. *J Rheumatol* 2000; **27**: 142-148
123 **Nakamura M**, Kondo H, Mori T, Komori A, Matsuyama M, Ito M, Takii Y, Koyabu M, Yokoyama T, Migita K, Daikoku M, Abiru S, Yatsuhashi H, Takezaki E, Masaki N, Sugi K, Honda K, Adachi H, Nishi H, Watanabe Y, Nakamura Y, Shimada M, Komatsu T, Saito A, Saoshiro T, Harada H, Sodeyama T, Hayashi S, Masumoto A, Sando T, Yamamoto T, Sakai H, Kobayashi M, Muro T, Koga M, Shums Z, Norman GL, Ishibashi

H. Anti-gp210 and anti-centromere antibodies are different risk factors for the progression of primary biliary cirrhosis. *Hepatology* 2007; **45**: 118-127
124 **Klein R**, Berg PA. Anti-M9 antibodies in sera from patients with primary biliary cirrhosis recognize an epitope of glycogen phosphorylase. *Clin Exp Immunol* 1990; **81**: 65-71
125 **Santiago M**, Baron M, Miyachi K, Fritzler MJ, Abu-Hakima M, Leclercq S, Bell M, Hudson M, Mathieu JP, Taillefer S, Jones N, Docherty P, Khraishi M, Markland J, Pope J, Robinson D, Smith D, Sutton E. A comparison of the frequency of antibodies to cyclic citrullinated peptides using a third generation anti-CCP assay (CCP3) in systemic sclerosis, primary biliary cirrhosis and rheumatoid arthritis. *Clin Rheumatol* 2008; **27**: 77-83
126 **Koga T**, Migita K, Miyashita T, Maeda Y, Nakamura M, Abiru S, Myoji M, Komori A, Yano K, Yatsuhashi H, Eguchi K, Ishibashi H. Determination of anti-cyclic citrullinated peptide antibodies in the sera of patients with liver diseases. *Clin Exp Rheumatol* 2008; **26**: 121-124
127 **Agmon-Levin N**, Shapira Y, Selmi C, Barzilai O, Ram M, Szyper-Kravitz M, Sella S, Katz BS, Youinou P, Renaudineau Y, Larida B, Invernizzi P, Gershwin ME, Shoenfeld Y. A comprehensive evaluation of serum autoantibodies in primary biliary cirrhosis. *J Autoimmun* 2010; **34**: 55-58
128 **Li L**, Chen M, Huang DY, Nishioka M. Frequency and significance of antibodies to chromatin in autoimmune hepatitis type I. *J Gastroenterol Hepatol* 2000; **15**: 1176-1182
129 **Czaja AJ**, Shums Z, Binder WL, Lewis SJ, Nelson VJ, Norman GL. Frequency and significance of antibodies to chromatin in autoimmune hepatitis. *Dig Dis Sci* 2003; **48**: 1658-1664
130 **Subasić D**, Karamehić J, Ljuca F, Gavrankapetanović F, Delić-Sarac M, Eminović I, Kovacević D. Correlation of autoantibodies presence detected by IFA-anti-dsDNA, IFA-AMA and immunoblotting with corresponding data in clinical management of autoimmune diseases. *Bosn J Basic Med Sci* 2008; **8**: 86-92
131 **Muratori P**, Granito A, Pappas G, Pendino GM, Quarneti C, Cicola R, Menichella R, Ferri S, Cassani F, Bianchi FB, Lenzi M, Muratori L. The serological profile of the autoimmune hepatitis/primary biliary cirrhosis overlap syndrome. *Am J Gastroenterol* 2009; **104**: 1420-1425
132 **Chou MJ**, Lee SL, Chen TY, Tsay GJ. Specificity of antinuclear antibodies in primary biliary cirrhosis. *Ann Rheum Dis* 1995; **54**: 148-151
133 **Tsuchiya K**, Kiyosawa K, Imai H, Sodeyama T, Furuta S. Detection of anti-double and anti-single stranded DNA antibodies in chronic liver disease: significance of anti-double stranded DNA antibody in autoimmune hepatitis. *J Gastroenterol* 1994; **29**: 152-158
134 **Konikoff F**, Swissa M, Shoenfeld Y. Autoantibodies to histones and their subfractions in chronic liver diseases. *Clin Immunol Immunopathol* 1989; **51**: 77-82
135 **Tishler M**, Alosachie I, Barka N, Lin HC, Gershwin ME, Peter JB, Shoenfeld Y. Primary Sjögren's syndrome and primary biliary cirrhosis: differences and similarities in the autoantibody profile. *Clin Exp Rheumatol* 1995; **13**: 497-500
136 **Granito A**, Muratori P, Muratori L, Pappas G, Cassani F, Worthington J, Ferri S, Quarneti C, Cipriano V, de Molo C, Lenzi M, Chapman RW, Bianchi FB. Antibodies to SS-A/Ro-52kD and centromere in autoimmune liver disease: a clue to diagnosis and prognosis of primary biliary cirrhosis. *Aliment Pharmacol Ther* 2007; **26**: 831-838
137 **Takada K**, Suzuki K, Matsumoto M, Okada M, Nakanishi T, Horikoshi H, Higuchi T, Ohsuzu F. Clinical characteristics of patients with both anti-U1RNP and anti-centromere antibodies. *Scand J Rheumatol* 2008; **37**: 360-364
138 **Dörner T**, Held C, Trebeljahr G, Lukowsky A, Yamamoto K, Hiepe F. Serologic characteristics in primary biliary cirrhosis associated with sicca syndrome. *Scand J Gastroenterol* 1994; **29**: 655-660
139 **Wesierska-Gadek J**, Penner E, Battezzati PM, Selmi C, Zuin M, Hitchman E, Worman HJ, Gershwin ME, Podda M, Invernizzi P. Correlation of initial autoantibody profile and clinical outcome in primary biliary cirrhosis. *Hepatology* 2006; **43**: 1135-1144
140 **Kanzler S**, Bozkurt S, Herkel J, Galle PR, Dienes HP, Lohse AW. [Presence of SLA/LP autoantibodies in patients with primary biliary cirrhosis as a marker for secondary autoimmune hepatitis (overlap syndrome)] *Dtsch Med Wochenschr* 2001; **126**: 450-456
141 **Eyraud V**, Chazouilleres O, Ballot E, Corpechot C, Poupon R, Johanet C. Significance of antibodies to soluble liver antigen/liver pancreas: a large French study. *Liver Int* 2009; **29**: 857-864
142 **Floreani A**, Baragiotta A, Leone MG, Baldo V, Naccarato R. Primary biliary cirrhosis and hepatitis C virus infection. *Am J Gastroenterol* 2003; **98**: 2757-2762
143 **McFarlane BM**, McSorley CG, Vergani D, McFarlane IG, Williams R. Serum autoantibodies reacting with the hepatic asialoglycoprotein receptor protein (hepatic lectin) in acute and chronic liver disorders. *J Hepatol* 1986; **3**: 196-205
144 **Bedlow AJ**, Donaldson PT, McFarlane BM, Lombard M, McFarlane IG, Williams R. Autoreactivity to hepatocellular antigens in primary biliary cirrhosis and primary sclerosing cholangitis. *J Clin Lab Immunol* 1989; **30**: 103-109
145 **Yoshioka M**, Mizuno M, Morisue Y, Shimada M, Hirai M, Nasu J, Okada H, Sakaguchi K, Yamamoto K, Tsuji T. Anti-asialoglycoprotein receptor autoantibodies, detected by a capture-immunoassay, are associated with autoimmune liver diseases. *Acta Med Okayama* 2002; **56**: 99-105
146 **Löhr H**, Treichel U, Poralla T, Manns M, Meyer zum Büschenfelde KH, Fleischer B. The human hepatic asialoglycoprotein receptor is a target antigen for liver-infiltrating T cells in autoimmune chronic active hepatitis and primary biliary cirrhosis. *Hepatology* 1990; **12**: 1314-1320
147 **Schuurman HJ**, Vogten AJ, Schalm SW, Fevery J. Clinical evaluation of the liver cell membrane autoantibody assay. *Digestion* 1982; **23**: 184-193
148 **Meliconi R**, Stancari MV, Garagnani M, Baraldini M, Stefanini GF, Miglio F, Gasbarrini G. Occurrence and significance of IgG liver membrane autoantibodies (LMA) in chronic liver diseases of different aetiology. *Clin Exp Immunol* 1983; **51**: 565-571
149 **Morichika S**, Arima T, Nagashima H. Clinical evaluation of autoantibodies to liver cell membrane specific antigen, liver specific lipoprotein, and Tamm-Horsfall glycoprotein in autoimmune chronic active hepatitis. *Gastroenterol Jpn* 1987; **22**: 24-33
150 **Meliconi R**, Facchini A, Miglio F, Trevisan A, Alberti A, Realdi G, Baraldini M, Gasbarrini G. Liver-specific autoantibodies in chronic hepatitis B virus infection. *Clin Sci* (Lond) 1985; **68**: 123-126
151 **Watanabe K**, Ohira H, Orikasa H, Saito K, Kanno K, Shioya Y, Obara K, Sato Y. Anti-calreticulin antibodies in patients with inflammatory bowel disease. *Fukushima J Med Sci* 2006; **52**: 1-11
152 **Abe K**, Ohira H, Kobayashi H, Saito H, Takahashi A, Rai T, Kanno Y, Monoe K, Watanabe H, Irisawa A, Sato Y. Breakthrough of immune self-tolerance to calreticulin induced by CpG-oligodeoxynucleotides as adjuvant. *Fukushima J Med Sci* 2007; **53**: 95-108
153 **Xia Q**, Lu F, Yan HP, Wang HX, Feng X, Zhao Y, Liu BY, Wang J, Li P, Xue Y, Hu MR, Qian L, Guo N, Yang SC, Li MY, Ma YF, Li BA, Zhang XM, Shen BF. Autoantibody profiling of Chinese patients with autoimmune hepatitis using immunoproteomic analysis. *J Proteome Res* 2008; **7**: 1963-1970
154 **Lu F**, Xia Q, Ma Y, Yuan G, Yan H, Qian L, Hu M, Wang M, Lu H, Wang H, Liu B, Xue Y, Wang H, Li M, Shen B, Guo N. Serum proteomic-based analysis for the identification of a potential serological marker for autoimmune hepatitis. *Biochem*

Biophys Res Commun 2008; **367**: 284-290
155 **Miyachi K**, Matsushima H, Hankins RW, Hirakata M, Mimori T, Hosaka H, Amagasaki Y, Miyakawa H, Kako M, Shibata M, Onozuka Y, Ueno U. A novel antibody directed against a three-dimensional configuration of a 95-kDa protein in patients with autoimmune hepatic diseases. *Scand J Immunol* 1998; **47**: 63-68
156 **Miyachi K**, Hirano Y, Horigome T, Mimori T, Miyakawa H, Onozuka Y, Shibata M, Hirakata M, Suwa A, Hosaka H, Matsushima S, Komatsu T, Matsushima H, Hankins RW, Fritzler MJ. Autoantibodies from primary biliary cirrhosis patients with anti-p95c antibodies bind to recombinant p97/VCP and inhibit in vitro nuclear envelope assembly. *Clin Exp Immunol* 2004; **136**: 568-573
157 **Miyachi K**, Hosaka H, Nakamura N, Miyakawa H, Mimori T, Shibata M, Matsushima S, Chinoh H, Horigome T, Hankins RW, Zhang M, Fritzler MJ. Anti-p97/VCP antibodies: an autoantibody marker for a subset of primary biliary cirrhosis patients with milder disease? *Scand J Immunol* 2006; **63**: 376-382
158 **Kato T**, Miyakawa H, Ishibashi M. Frequency and significance of anti-glutathione S-transferase autoantibody (anti-GST A1-1) in autoimmune hepatitis. *J Autoimmun* 2004; **22**: 211-216
159 **Pelli N**, Fensom AH, Slade C, Boa F, Mieli-Vergani G, Vergani D. Argininosuccinate lyase: a new autoantigen in liver disease. *Clin Exp Immunol* 1998; **114**: 455-461
160 **Ikeda Y**, Toda G, Hashimoto N, Aotsuka S, Yokohari R, Maruyama T, Oka H. Anticalmodulin autoantibody in liver diseases: a new antibody against a cytoskeleton-related protein. *Hepatology* 1987; **7**: 285-293
161 **Sakly W**, Jeddi M, Ghedira I. Anti-Saccharomyces cerevisiae antibodies in primary biliary cirrhosis. *Dig Dis Sci* 2008; **53**: 1983-1987
162 **Fagoonee S**, De Luca L, De Angelis C, Castelli A, Rizzetto M, Pellicano R. Anti-Saccharomyces cerevisiae as unusual antibodies in autoimmune hepatitis. *Minerva Gastroenterol Dietol* 2009; **55**: 37-40
163 **Muratori P**, Muratori L, Guidi M, Maccariello S, Pappas G, Ferrari R, Gionchetti P, Campieri M, Bianchi FB. Anti-Saccharomyces cerevisiae antibodies (ASCA) and autoimmune liver diseases. *Clin Exp Immunol* 2003; **132**: 473-476
164 **Jensen-Jarolim E**, Neumann C, Oberhuber G, Gscheidlinger R, Neuchrist C, Reinisch W, Zuberi RI, Penner E, Liu FT, Boltz-Nitulescu G. Anti-Galectin-3 IgG autoantibodies in patients with Crohn's disease characterized by means of phage display peptide libraries. *J Clin Immunol* 2001; **21**: 348-356
165 **Bizzaro N**, Tampoia M, Villalta D, Platzgummer S, Liguori M, Tozzoli R, Tonutti E. Low specificity of anti-tissue transglutaminase antibodies in patients with primary biliary cirrhosis. *J Clin Lab Anal* 2006; **20**: 184-189
166 **Chatzicostas C**, Roussomoustakaki M, Drygiannakis D, Niniraki M, Tzardi M, Koulentaki M, Dimoulios P, Mouzas I, Kouroumalis E. Primary biliary cirrhosis and autoimmune cholangitis are not associated with coeliac disease in Crete. *BMC Gastroenterol* 2002; **2**: 5
167 **Volta U**, De Franceschi L, Molinaro N, Cassani F, Muratori L, Lenzi M, Bianchi FB, Czaja AJ. Frequency and significance of anti-gliadin and anti-endomysial antibodies in autoimmune hepatitis. *Dig Dis Sci* 1998; **43**: 2190-2195
168 **Roozendaal C**, de Jong MA, van den Berg AP, van Wijk RT, Limburg PC, Kallenberg CG. Clinical significance of anti-neutrophil cytoplasmic antibodies (ANCA) in autoimmune liver diseases. *J Hepatol* 2000; **32**: 734-741
169 **Sundewall AC**, Lefvert AK, Norberg R. Characterization of anti-acetylcholine receptor antibody activity in patients with anti-mitochondrial antibodies. *Clin Immunol Immunopathol* 1987; **45**: 184-195
170 **Ohana M**, Okazaki K, Hajiro K, Uchida K. Antilactoferrin antibodies in autoimmune liver diseases. *Am J Gastroenterol* 1998; **93**: 1334-1339
171 **Muratori L**, Muratori P, Zauli D, Grassi A, Pappas G, Rodrigo L, Cassani F, Lenzi M, Bianchi FB. Antilactoferrin antibodies in autoimmune liver disease. *Clin Exp Immunol* 2001; **124**: 470-473
172 **Gabeta S**, Norman GL, Gatselis N, Liaskos C, Papamichalis PA, Garagounis A, Zachou K, Rigopoulou EI, Dalekos GN. IgA anti-b2GPI antibodies in patients with autoimmune liver diseases. *J Clin Immunol* 2008; **28**: 501-511
173 **Zachou K**, Liaskos C, Rigopoulou E, Gabeta S, Papamichalis P, Gatselis N, Georgiadou S, Dalekos GN. Presence of high avidity anticardiolipin antibodies in patients with autoimmune cholestatic liver diseases. *Clin Immunol* 2006; **119**: 203-212
174 **Pietropaolo M**, Peakman M, Pietropaolo SL, Zanone MM, Foley TP Jr, Becker DJ, Trucco M. Combined analysis of GAD65 and ICA512(IA-2) autoantibodies in organ and non-organ-specific autoimmune diseases confers high specificity for insulin-dependent diabetes mellitus. *J Autoimmun* 1998; **11**: 1-10
175 **Fida S**, Myers MA, Whittingham S, Rowley MJ, Ozaki S, Mackay IR. Autoantibodies to the transcriptional factor SOX13 in primary biliary cirrhosis compared with other diseases. *J Autoimmun* 2002; **19**: 251-257
176 **Nakamura H**, Usa T, Motomura M, Ichikawa T, Nakao K, Kawasaki E, Tanaka M, Ishikawa K, Eguchi K. Prevalence of interrelated autoantibodies in thyroid diseases and autoimmune disorders. *J Endocrinol Invest* 2008; **31**: 861-865
177 **Fusconi M**, Vannini A, Dall'Aglio AC, Pappas G, Cassani F, Ballardini G, Frisoni M, Grassi A, Bianchi FB, Zauli D. Anti-cyclic citrullinated peptide antibodies in type 1 autoimmune hepatitis. *Aliment Pharmacol Ther* 2005; **22**: 951-955
178 **Mayo I**, Arizti P, Parés A, Oliva J, Doforno RA, de Sagarra MR, Rodés J, Castaño JG. Antibodies against the COOH-terminal region of E. coli ClpP protease in patients with primary biliary cirrhosis. *J Hepatol* 2000; **33**: 528-536
179 **Bogdanos DP**, Baum H, Sharma UC, Grasso A, Ma Y, Burroughs AK, Vergani D. Antibodies against homologous microbial caseinolytic proteases P characterise primary biliary cirrhosis. *J Hepatol* 2002; **36**: 14-21
180 **Roesler KW**, Schmider W, Kist M, Batsford S, Schiltz E, Oelke M, Tuczek A, Dettenborn T, Behringer D, Kreisel W. Identification of beta-subunit of bacterial RNA-polymerase--a non-species-specific bacterial protein--as target of antibodies in primary biliary cirrhosis. *Dig Dis Sci* 2003; **48**: 561-569
181 **Takiguchi J**, Ohira H, Rai T, Abe K, Takahashi A, Sato Y. Anti-eosinophil peroxidase antibodies detected in patients with primary biliary cirrhosis. *Hepatol Res* 2005; **32**: 33-37
182 **Herkel J**, Modrow JP, Bamberger S, Kanzler S, Rotter V, Cohen IR, Lohse AW. Prevalence of autoantibodies to the p53 protein in autoimmune hepatitis. *Autoimmunity* 2002; **35**: 493-496
183 **Kyriatsoulis A**, Manns M, Gerken G, Lohse AW, Maelicke A, Wessler I, Reske K, Meyer zum Büschenfelde KH. Immunochemical characterization of anti-acetylcholine receptor antibodies in primary biliary cirrhosis. *J Hepatol* 1988; **6**: 283-290
184 **Sundewall AC**, Lefvert AK, Olsson R. Anti-acetylcholine receptor antibodies in primary biliary cirrhosis. *Acta Med Scand* 1985; **217**: 519-525
185 **Invernizzi P**, Battezzati PM, Crosignani A, Zermiani P, Bignotto M, Del Papa N, Zuin M, Podda M. Antibody to carbonic anhydrase II is present in primary biliary cirrhosis (PBC) irrespective of antimitochondrial antibody status. *Clin Exp Immunol* 1998; **114**: 448-454
186 **Ueno Y**, Ishii M, Igarashi T, Mano Y, Yahagi K, Kisara N, Kobayashi Y, Niitsuma H, Kobayashi K, Shimosegawa T. Primary biliary cirrhosis with antibody against carbonic anhydrase II associates with distinct immunological backgrounds. *Hepatol Res* 2001; **20**: 18-27
187 **Comay D**, Cauch-Dudek K, Hemphill D, Diamandis E, Wan-

less I, Heathcote EJ. Are antibodies to carbonic anhydrase II specific for anti-mitochondrial antibody-negative primary biliary cirrhosis? *Dig Dis Sci* 2000; **45**: 2018-2021
188 **Hosoda H**, Okawa-Takatsuji M, Tanaka A, Uwatoko S, Aotsuka S, Hasimoto N, Ozaki Y, Ikeda Y. Detection of autoantibody against carbonic anhydrase II in various liver diseases by enzyme-linked immunosorbent assay using appropriate conditions. *Clin Chim Acta* 2004; **342**: 71-81
189 **Alver A**, Menteşe A, Karahan SC, Erem C, Keha EE, Arikan MK, Eminağaoğlu MS, Deger O. Increased serum anti-carbonic anhydrase II antibodies in patients with Graves' disease. *Exp Clin Endocrinol Diabetes* 2007; **115**: 287-291
190 **Akisawa N**, Maeda T, Iwasaki S, Onishi S. Identification of an autoantibody against alpha-enolase in primary biliary cirrhosis. *J Hepatol* 1997; **26**: 845-851
191 **Saulot V**, Vittecoq O, Charlionet R, Fardellone P, Lange C, Marvin L, Machour N, Le Loët X, Gilbert D, Tron F. Presence of autoantibodies to the glycolytic enzyme alpha-enolase in sera from patients with early rheumatoid arthritis. *Arthritis Rheum* 2002; **46**: 1196-1201
192 **Bogdanos DP**, Gilbert D, Bianchi I, Leoni S, Mitry RR, Ma Y, Mieli-Vergani G, Vergani D. Antibodies to soluble liver antigen and alpha-enolase in patients with autoimmune hepatitis. *J Autoimmune Dis* 2004; **1**: 4
193 **Mosca M**, Chimenti D, Pratesi F, Baldini C, Anzilotti C, Bombardieri S, Migliorini P. Prevalence and clinico-serological correlations of anti-alpha-enolase, anti-C1q, and anti-dsDNA antibodies in patients with systemic lupus erythematosus. *J Rheumatol* 2006; **33**: 695-697
194 **Terrier B**, Degand N, Guilpain P, Servettaz A, Guillevin L, Mouthon L. Alpha-enolase: a target of antibodies in infectious and autoimmune diseases. *Autoimmun Rev* 2007; **6**: 176-182
195 **Vermeulen N**, Arijs I, Joossens S, Vermeire S, Clerens S, Van den Bergh K, Michiels G, Arckens L, Schuit F, Van Lommel L, Rutgeerts P, Bossuyt X. Anti-alpha-enolase antibodies in patients with inflammatory Bowel disease. *Clin Chem* 2008; **54**: 534-541
196 **Yamaguchi H**, Miura H, Ohsumi K, Ishimi N, Taguchi H, Ishiyama N, Shiraishi Y, Yamamoto T, Ogata S. Detection and characterization of antibodies to bacterial heat-shock protein 60 in sera of patients with primary biliary cirrhosis. *Microbiol Immunol* 1994; **38**: 483-487
197 **Martins EB**, Chapman RW, Marron K, Fleming KA. Biliary expression of heat shock protein: a non-specific feature of chronic cholestatic liver diseases. *J Clin Pathol* 1996; **49**: 53-56
198 **Vilagut L**, Parés A, Viñas O, Vila J, Jiménez de Anta MT, Rodés J. Antibodies to mycobacterial 65-kD heat shock protein cross-react with the main mitochondrial antigens in patients with primary biliary cirrhosis. *Eur J Clin Invest* 1997; **27**: 667-672
199 **Bogdanos DP**, Pares A, Baum H, Caballeria L, Rigopoulou EI, Ma Y, Burroughs AK, Rodes J, Vergani D. Disease-specific cross-reactivity between mimicking peptides of heat shock protein of Mycobacterium gordonae and dominant epitope of E2 subunit of pyruvate dehydrogenase is common in Spanish but not British patients with primary biliary cirrhosis. *J Autoimmun* 2004; **22**: 353-362
200 **Shinkura N**, Ikai I, Yamauchi A, Hirose T, Kawai Y, Inamoto T, Ozaki S, Iwai M, Bona C, Yamaoka Y. Autoantibodies to FK506 binding protein 12 (FKBP12) in autoimmune diseases. *Autoimmunity* 1999; **29**: 159-170

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TOPIC HIGHLIGHT

Peter V Draganov, Dr., MD, Associate Professor, *Series Editor*

Colonoscopic polypectomy and associated techniques

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Abstract

Polypectomy of colonic polyps has been shown to reduce the risk of colon cancer development and is considered a fundamental skill for all endoscopists who perform colonoscopy. A variety of polypectomy techniques and devices are available, and their use can vary greatly based on local availability and preferences. In general, cold forceps and cold snare have been the polypectomy methods of choice for smaller polyps, and hot snare has been the method of choice for larger polyps. The use of hot forceps has mostly fallen out of favor. Polypectomy for difficult to remove polyps may require the use of special devices and advanced techniques and has continued to evolve. As a result, the vast majority of polyps today can be removed endoscopically. Since electrocautery is frequently used for polypectomy, endoscopists should be thoroughly familiar with the basic principles of electrosurgery as it pertains to polypectomy. Tattooing of a polypectomy site is an important adjunct to polypectomy and can greatly facilitate future surgery or endoscopic surveillance. The two most common post-polypectomy complications are bleeding and perforation. Their incidence can be decreased with the use of meticulous polypectomy techniques and the application of some prophylactic maneuvers. This review will examine the technique of polypectomy and its complications from the perspective of the practicing gastroenterologist.

Key words: Colonic polyp; Polypectomy; Colonoscopy; Polypectomy technique; Complications

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INTRODUCTION

Polypectomy is a fundamental skill utilized by all endoscopists who perform colonoscopy. Mastery of polypectomy is difficult and requires both significant experience and study. It is clear that polypectomy is efficacious in reducing the risk of colon cancer development by interrupting the adenoma to carcinoma progression[1,2]. Endoscopic techniques used in colonoscopic polypectomy continue to evolve, and it is important for all endoscopists to be familiar with these concepts.

Decision making about how to perform polypectomy is often made during colonoscopy when a polyp is detected. A general rule is that all potential adenomas should be removed. The endoscopic appearance of a polyp is often not necessarily a good indicator of its histologic nature. While as many as 70% of diminutive polyps (less

than 5 mm) may be adenomas, the risk of any particular polyp containing malignancy increases with the size of the polyp[3-6]. The method chosen for polypectomy is often related to the appearance and size of the polyp. Polyps are usually described as being pedunculated, sessile or flat. The risk of a polyp 2 cm in size or larger being malignant is greater than 10%[7]. Some polyps blur the lines though, by not falling into these strict categories. Nevertheless, consideration of polyp characteristics is helpful in determining the best approach to polypectomy[8].

COLD FORCEPS POLYPECTOMY

The simplest method for polypectomy is cold forceps removal. A survey of common practices among gastroenterologists found that cold forceps polypectomy was the technique of choice for small polyps, particularly polyps 1 to 3 mm in size[9]. In slightly larger polyps, jumbo forceps could be considered. Cold forceps can easily grasp small polyps that otherwise might be too small to snare.

After passing the forceps through the channel, the forceps and the scope can be manipulated in order to grasp as much polyp tissue as possible. Turning the scope to bring the polyp to the five to seven o'clock position can be useful since that is the position at which the forceps exit the endoscope channel. After closing the forceps on the polyp, a gentle pull on the wire removes the bite of polyp from the colon mucosa. The area is examined to determine if further bites are necessary to complete polyp excision.

Advantages to cold forceps polypectomy include avoiding risk associated with electrocautery and an almost negligible risk of colonic perforation[10]. One challenge associated with cold forceps polypectomy is that after the initial bite, minor bleeding can obscure the polypectomy field increasing the risk of leaving residual polyp behind[11].

HOT FORCEPS POLYPECTOMY

Hot forceps polypectomy is another option for small polyps. Hot forceps polypectomy is similar to cold forceps except it uses electrocautery to try to destroy residual polyp tissue intentionally left behind[12]. In hot forceps polypectomy, only the tip of the polyp is grabbed in the forceps. The small polyp is pulled into the colon lumen to create a tent-like effect and electrocautery is applied to destroy the polyp base while preserving the polyp tissue inside the forceps as a histological specimen[13].

Over the years, the use of hot forceps has fallen out of favor. One randomized study by Ellis looked at 72 polyps 6 mm or less in size and found that hot forceps still left residual polyp tissue behind 22% of the time compared to only 5%-14% of the time with either cold or hot snare[14]. Another study by Peluso retrospectively looked at 62 hot forceps polypectomies for polyps 3-6 mm in size and found that 17% of the time residual polyp tissue remained on follow-up endoscopic exam 1 to 2 wk later[15]. Cold forceps and snare polypectomy have been described as having a 16% residual polyp rate which suggests that hot forceps are either no better or even worse than other accepted methods of polyp removal. Hot forceps may still be useful though for small polyps that have a tip easily grasped with forceps but a polyp base that is hard to reach yet could still be destroyed with application of electrocautery.

SNARE POLYPECTOMY

Snare polypectomy was found to be the preferred method for removal of polyps 1 cm or greater in size in a survey of common gastroenterology practices[9]. A snare is a self-contained metal ring that is opened over the polyp and then closed entrapping polyp tissue for resection by closing the ring. Before pulling the snare out of the scope, the polyp should be brought to the six o'clock position. Sometimes advancing the snare proximal to (beyond) the polyp is useful if the polyp is behind a fold or inclined to flop out of the opened snare. The snare can also be used to position a pedunculated polyp in such a position as is more amenable to capturing once the snare is opened. Once the polyp is captured in the snare, the snare plastic sheath should be advanced moving the polyp away from the scope tip if electrocautery is to be used to avoid electrical damage to the scope. When snaring a pedunculated polyp, the snare should be placed about half way up the stalk, so that after cutting, a stalk remnant is left which can be grabbed or clipped if hemorrhage occurs. The polyp is pulled away from its base into the lumen tenting the colon wall to avoid burning the adjacent deep colon layers[11].

A snare can be either hot or cold in that it can be supplemented with electrocautery or not. During hot snaring, the endoscopist's assistant should close the snare slowly and gently. If the snare is too tight prior to electrocautery application, it could result in inadvertent cold cutting the polyp, resulting in bleeding from the stalk or in the snare becoming entrapped into coagulated tissue in the stalk[16]. Once the snare is in position, a few seconds of electrocautery can be applied if opted for, and then the endoscopist instructs the assistant to cut through the polyp.

There are many different types of snares each with specific advantages which can be chosen depending on the situation. Oval and hexagonal snares are most commonly used. We suggest using a barbed snare for hard to grab tissue as can be the case in flat or sessile polyps or when the snare slipping off the polyp seems to be a problem. Crescent snares are often used in EMR. A rotatable snare is useful when initially the snare comes out of the scope in such a way that is not optimal for snaring the polyp and it is desirable to rotate the snare to an angle that is better for capturing the polyp. A mini-snare can be used for cold snaring smaller polyps or to remove a small amount of residual tissue after piecemeal polypectomy[17]. There is a combination snare-injection needle which allows for quick injection prior to opening the snare and avoids having to change out an injection needle wire for

the snare (i-Snare system, US endoscopy, Mentor, Ohio, USA)[18].

ELECTROCAUTERY

The purpose of electrocautery in polypectomy is to either provide extra power in cutting tissue or to prevent bleeding by coagulation of tissue. The basic principle in electrocautery is that if enough electrical current is delivered, heat will be generated to cause cellular bursting leading to tissue cutting. If somewhat less heat is generated then cell shrinkage leading to tissue coagulation occurs. Even pure cut current causes some coagulation, and pure coagulation current has some cutting property. Snares and hot forceps use monopolar electrocautery, which means that the electrical circuit runs through the patient body to a grounding pad placed on the patient. Cautery probes can also use bipolar electrocautery, which means that the electrical circuit runs between two electrodes both located on the tip of the probe. Energy deliverance is also proportional to the time it is applied, so the length of time the endoscopist keeps their foot on the pedal is very important[16]. The use of coagulation current has been associated with more delayed post-polypectomy hemorrhage, whereas the use of cutting and blended current have been associated with more immediate hemorrhage[19]. A review of electrocautery by Morris suggests using coagulation at a setting of 20 Watts for hot snaring. Since cut has also been associated with a higher risk of perforation, we suggest first using coagulation for standard colonoscopic snare polypectomy. Then after using coagulation, the endoscopist can consider using some cut function next if the polyp has a thick stock and coagulation alone is unable to cut through it or in the case that the snare becomes entrapped on the polyp stock. For hot forceps electrocautery coagulation at 10-20 Watts can be used[16]. Most modern electrosurgical units have preset polypectomy settings.

LARGE POLYPS

In the past, large polyps often required surgery for removal, but now many can be managed endoscopically[20]. Endoscopic Mucosal Resection (EMR) can be performed on sessile polyps 2 cm in size or larger. EMR involves submucosal injection (often of saline) creating a cushion for the polyp and then hot snaring the polyp either *en bloc* (all together) or piecemeal (multiple snarings). EMR can provide resection down to the muscularis propria[21-23]. There is no official distinction between saline assisted piece meal polypectomy and EMR but typically the term polypectomy is reserved for removal of flat lesions measuring less than 2 cm and the term EMR is used for larger lesions[4,24]. Endoscopic Submucosal Dissection aims to remove all dysplastic tissue en-block as one piece rather than the piecemeal technique that is used with saline assisted polypectomy and EMR[25]. Large polyps are often adenomatous, therefore complete resection is the goal even though it is often time-consuming. Iishi found that 55% of polyps resected in piecemeal fashion required further resection on a repeat colonoscopy, but complete resection was possible in 83% of polyps after up to three repeat colonoscopies[26]. Flat and sessile polyps can be challenging to snare as they are often level with the colon floor. The first piece of tissue snared can leave divots or ledges in the remaining polyp that can make it more easily grabbed in subsequent snares. If residual polyp tissue is left after piecemeal polypectomy, argon plasma coagulation (APC) can be used to tryw to destroy the residual tissue[27]. After any piecemeal polypectomy, the site should be re-examined in 2 to 6 mo to evaluate for any residual polyp tissue[7,8].

POLYP RETRIEVAL

Once polyp tissue is snared, actually retrieving it can be challenging. Many endoscopists periodically experience the frustration of successfully snaring a large adenoma-appearing polyp only for it to fall out of view or get lost in the colon somewhere[28]. However, even experienced endoscopists may fail to retrieve polyp tissue up to 16% of the time[8]. Possibly the most common way to retrieve a polyp once it is snared is to drive the scope up to the polyp in the six o'clock position and then to suction the polyp through the scope into a trap, using a back flush if needed. If the polyp is too big to be suctioned into the scope, the snare can be used to cut the large polyp into pieces small enough to fit through the suction channel. Polyp tissue can also be grabbed with forceps while the entire colonoscope is withdrawn. In these cases the forceps can be advanced out a few centimeters so that simultaneous examination of the remaining colon can be performed while the specimen is kept in view. A Roth net can be used to remove large polyps or several polyp fragments at once. Also, an overtube can be used for easy repeated colonoscopic intubation to the polypectomy site with repeated removal of polyp fragments[29,30].

RESIDUAL POLYP TISSUE

Leaving residual polyp tissue behind leaves behind cells that may continue to progress through the adenoma to carcinoma sequence, therefore the purpose of polypectomy is to break that sequence. Risk of residual polyp tissue is often the outcome measured in studies comparing different methods of polypectomy, such as snare *vs* forceps. In an observational study, Tappero *et al*[17] found that a snare never left behind residual polyp tissue but cold forceps often did. Zlatanic found that for treating residual tissue, piecemeal polypectomy left behind residual tissue 46% of the time, APC destruction still left residual tissue 50% of the time, and doing nothing left behind residual polyp obviously 100% of the time[24].

THE CHALLENGING POLYPECTOMY

Some polyps provide distinct challenges that call for utilizing other approaches than just standard polypectomy techniques. Endoscopists periodically find polyps that

are very difficult to remove. These can include polyps that are located behind colon folds, polyps that are very large, polyps that are just out of reach, and flat, carpeted, or thick polyps. For polyps hiding behind folds and large pedunculated polyps, Valentine *et al*[31] described a technique using a double channel therapeutic endoscope. A tripronged grasper is advanced *via* one of the channels to pull the polyp into better view and into the snare, while a snare for polypectomy is inserted through the other channel. A standard upper endoscope can also be considered for difficult to reach polyps as it has a tip with a tighter bending angle than a colonoscope[32]. A side viewing scope can be used for polyps that are behind folds or on a side of the colon wall unable to be reached by a standard colonoscope. Friedland[33] described either retroflexing the colonoscope or injecting a large amount of saline proximal to the lesion as options to try to reach polyps on the inside wall of tight turns. Even two different scopes manipulated by two endoscopists can be attempted with one scope grabbing the polyp and pulling it into a convenient location while the other scope performs polypectomy has been described[8]. Colon spasms can present a challenge by constantly moving the polyp in and out of view, and glucagon can be given intravenously to decrease these spasms[8]. Some polyps may not be amenable to endoscopic polypectomy and are better served with surgery. If a large polyp is in the cecum, extends into the ileocecal valve, or extends into the appendix, surgery may need to be considered. Also polyps that involve more than 30% of the colon circumference are often impossible to remove endoscopically[11].

Injection

An important related tool to consider for polypectomy is injection with either saline or epinephrine (1:10000) into the polyp base or stalk. The submucosa is the target location for fluid deposit, so the endoscopist should try not to penetrate the colon wall with the needle. Injected fluid can diffuse fast, so sometimes repeat injections are needed. Injection is suggested in the literature for larger polyps specifically. Most studies looking at resection of large or giant polyps include epinephrine injection in their polypectomy protocol. Injection can lift up flatter polyps rendering them more polypoid and more amenable to snare polypectomy and complete resection[34]. The injected fluid may also serve as a safety cushion by increasing the distance between the mucosa and the muscle layer and serosa, thereby at least theoretically decreasing risk of perforation[21,35]. If a polyp does not lift with an appropriate injection technique it may be caused by an underlying cancer extending to deeper colon layers. Pedunculated polyps with large stalks are more inclined to bleeding. Injecting these large stalks before snare polypectomy may provide prophylactic hemostasis and reduce the risk of a post-polypectomy bleed. Epinephrine is a potent vasoconstrictor, and both saline and epinephrine can exert a tamponade effect on blood vessels[36]. A study by Dobrowolski randomized 100 polyps to either epinephrine injection or no injection and found one post-polypectomy bleed in the injection group compared to 8 bleeds in the no injection group[37].

Endoloops

In addition to injection, another option for prevention of post-polypectomy bleed is an endoloop[38]. The endoloop is a detachable oval-shaped nylon snare. It is deployed in the same way as a standard snare but then tightened and released around the stalk or base of the polyp prior to polypectomy. A gastroenterology survey showed that 38% of endoscopists report using endoloops[9]. A trial done in Greece by Kouklakis randomized 64 patients with polyps greater than 2 cm in size to get either epinephrine injection or a combination of endoloop and endoclip placement. The combination endoloop and clip group did significantly better with only 3% post-polypectomy hemorrhages compared to the epinephrine group which had a 12% rate of post-polypectomy hemorrhage[39]. The Di Giorgio study found a lower rate of post-polypectomy bleed at 1.8% with a detachable snare compared to 3% for epinephrine injection and 8% for no prevention[40]. In 152 snare polypectomies, Paspatis *et al*[41] found that combination epinephrine injection with endoloop placement was associated with only a 1% rate of delayed bleeding whereas epinephrine used alone was associated with an 11% rate of delayed bleeding. However many problems with endoloops such as slipping off the polyp stalk, inadequate tightening, and persistence of bleeding despite endoloop placement were described in a retrospective study by Matsushita *et al*[42].

Tattooing

Large or polyps suspicious for invasive cancer should be considered for tattooing for easier future localization either by a surgeon during colectomy or by an endoscopist during future surveillance colonoscopy[43,44]. Endoclip placement and inter-operative colonoscopy are other ways to re-identify a lesion, however the endoclips can slip off prior to surgery, and inter-operative colonoscopy can be cumbersome and time-consuming. India ink is the preferred identification agent for tattooing polyps[45] because the ink is phagocytosed by macrophages giving the site an almost permanent easily detected marking. Other dyes like indigo carmine and methylene blue are too rapidly resorbed to be useful. Commercially available India ink is a sterile carbon based dye suspended in stabilizing particles and diluted in normal saline to a 1:100 concentration[46]. India ink is injected through an injection needle and targeted to the submucosal layer of the inter-haustral folds. Common practice is to place a tattoo on more than one side of the lesion in either a two or a four quadrant manner. Injecting at an oblique angle tangential to the colon wall can avoid penetration of the colon wall which can result in inflammation and a diffuse staining of the peritoneum thereby obscuring the surgeon's view during operation[44,47]. To ensure proper ink placement, a double injection technique has been described in which 1 mL of saline is first injected creating a submucosal bleb[48]. Once the

saline bleb is made, the needle is left in place, the saline syringe is changed to an India ink syringe and about 0.1 to 2 mL of tattoo ink is then injected into the bleb space[49,50]. After tattooing the polyp site, the endoscopist should also include in the report the distance of the site from the anal verge in centimeters to aid in future localization.

ENHANCED POLYP DETECTION AND CLASSIFICATION TECHNIQUES

Standard colonoscopy based on white light may have a polyp miss rate of anywhere from 1% to 26%[51]. Also distinguishing truly neoplastic lesions from normal or benign tissue endoscopically can be challenging. Potentially unnecessary biopsies require pathologic evaluation leading to increased costs, so one advantage of enhanced detection techniques includes avoiding this increased cost[52]. Some newer modes of enhanced polyp detection and classification have been developed over the last few years. High definition colonoscopy, chromoendoscopy, and narrow band imaging (NBI) are useful to enhance polyp detection. Confocal Laser Endoscopy, and spectroscopic colonoscopy are more for enhancing polyp classification.

High definition colonoscopy (complete system can cost $215 000 from Olympus America, Center Valley, PA, USA) provides an image containing more pixels and better picture quality than standard definition colonoscopy. One retrospective study by Buchner showed a significantly higher polyp and adenoma detection rate (4%-5% increase in yield) with high definition colonoscopy compared to standard definition colonoscopy for polyps less than 1 cm and in the left colon[53].

Another enhanced detection technique, chromoendoscopy, uses indigo carmine (25 g costs about $40) that is flushed over the colonic mucosa to demarcate polyp architecture, vascular pattern and pit detail. This can highlight subtle differences between normal colonic tissue and polyp tissue making polyp detection easier. NBI, a type of virtual chromoendoscopy, is another enhanced mode that uses special narrow band filters to enhance surface and vascular pattern appearance of potential polyps. NBI may be useful for distinguishing between hyperplastic and adenomatous polyps as well. One study from Japan looked at NBI in the evaluation of 617 colorectal lesions and reported a sensitivity of 90.9% and a specificity of 97.1% for differentiating non-neoplastic from hyperplastic lesions[54]. Round and stellate pit patterns represent benign lesions, and villiform, gyrus-like, and irregular patterns represent neoplastic lesions. Many standard colonoscopies now have NBI capability (which means no additional cost to patients when it is used) which is activated by pushing a button on the head of the scope[55,56].

A new spectroscopic probe (not commercially available yet) has been developed that detects the increased microvascular blood supply in normal tissue at the periphery of a polyp that may be unseen or behind a fold. This alerts the endoscopist "like a metal detector going off" to examine the nearby mucosa more carefully to find the polyp thereby increasing detection[57].

Confocal laser endoscopy (CLE) is an enhanced mode of polyp classification (Cellvizio, Paris, France). Once a potential polyp is endoscopically detected, the lesion is focused on for analysis to determine if it is benign or neoplastic. Thousands of optical fibers bundled together take 12 pictures per second and provide image resolution detailed to the micron level. Pit pattern, crypt architecture, and vascular patters are analyzed; and irregular vessels, presence of mucin and increased tissue density indicate a neoplastic lesion. CLE is either integrated into the scope or used as a separate probe passed through the accessory channel[58]. A study from Mayo Jacksonville found CLE to have a sensitivity of 76% and a specificity of 72% in differentiating non-neoplastic from neoplastic lesions. Interobserver agreement over what the images represented was found to be 78%[59].

HEMORRHAGE

Even though the benefit of polypectomy is significant in terms of reducing the risk of colon cancer development, polypectomy is not without some risk of complications. Most complications are related either to post-polypectomy hemorrhage or perforation. Hemorrhage is the most common and is usually divided into immediate (less than 12 h post-procedure) and delayed (after 12 h post-procedure but up to 30 d). There is a greater risk of immediate hemorrhage associated with cut or blended electrocautery and a greater risk of delayed hemorrhage with the use of coagulation current. These specific risks should be appreciated and weighed when choosing electrocautery type.

Dobrowolski *et al*[60] noted that the risk of post-polypectomy hemorrhage ranges from 0.3% to 6% but can be as high as 24% in large polyps. He found that hemorrhage was more likely in polyps larger than 17 mm, pedunculated polyps with stalks thicker than 5 mm, sessile polyps, and malignant polyps. Watabe found that hypertension also puts patients at risk for a delayed post-polypectomy hemorrhage[61].

Immediate hemorrhages are frequently noticed during colonoscopic examination as bleeding from the polypectomy site is directly visualized. In these cases, either epinephrine injection into the base of the polypectomy site or endoclip placement is often considered as first line hemostatic therapy. Endoloop placement can also be considered and applied either to a stalk or to a larger polypectomy base for hemostasis. If snaring a pedunculated polyp results in a visibly bleeding stalk, sometimes grasping the stalk with the snare and holding pressure for 5 min can stop the hemorrhage[36].

Endoclips can be placed onto a bleeding residual stalk or empirically placed just lateral to the polypectomy site to tamponade any supplying blood vessels[62]. Endoclips can also be placed prophylactically at the polypectomy site after removal of the polyp. A group in Spain looked retrospectively at 34 polypectomies using endo-

clips either before or after resection of polyps 15-40 mm in size with stalks 5-12 mm in thickness. They found that all episodes of bleeding could be controlled with the use of endoclips. They also found that the clips easily catch stalks around 5 mm in thickness but that two clips could be placed on stalks thicker than that[63].

Friedland *et al*[64] described performing polypectomy on polyps less than 1 cm in size in actively anticoagulated patients. He placed endoclips prophylactically at the polypectomy site and had no more incidence of post-polypectomy bleed than in non-anticoagulated patients.

Many forceps polypectomies result in some minor oozing from capillaries at the polypectomy site, and this is usually self-limited and resolves after continued visualization. Delayed hemorrhage can require hospitalization, blood transfusion, and repeat colonoscopy for definitive hemostasis.

PERFORATION

Perforation is a serious complication that can result from polypectomy and can often have major clinical ramifications for the patient after the procedure is over[65]. Factors contributing to perforation include mechanical stress from the scope, barotrauma, electrocautery, and the depth of the polyp resection itself. The risk of perforation with all colonoscopies has been estimated somewhere around 1 perforation per 1000 to 2000 colonoscopies[66-68]. Risk of perforation however increases in polypectomies involving longer electrocautery time, removal of larger polyps, location in the cecum, and large sessile polyps requiring piecemeal removal.

If a perforation is visualized during the procedure itself, the endoscopist can consider an attempt at closure with endoclips. The progress of Natural Orifice Translumenal Endoscopic Surgery research has highlighted the reality of closing a perforation endoscopically with endoclips[69]. However, emergency computed tomography imaging, antibiotic administration, bowel rest and surgical consultation still play an important role. Unfortunately, approximately 5% of perforations result in patient death[11].

Similar to perforation but less serious is post-polypectomy syndrome, another complication where there is a transmural burn not resulting in perforation. Post-polypectomy syndrome presents with leukocytosis, fever and abdominal pain in the absence of free air on imaging. Treatment of post-polypectomy syndrome is usually conservative involving antibiotics, fluids, and bowel rest[8].

CONCLUSION

In summary, colonoscopic polypectomy is a continuously evolving therapy that has been remarkable at reducing the risk of colorectal cancer. Gastroenterologists must be thoughtful and proficient in techniques such as snaring, injection, tattooing, and all other tools related to polypectomy for endoscopic success. Cold forceps seem to be preferred for small polyps and snares for larger. Coagulation current may be the electrocautery mode of choice for polypectomy, although it is associated with higher risk of delayed hemorrhage. Difficult to reach polyps continue to require various endoscopic tricks and an ability to improvise for successful resection. There are several options for prevention of bleeding in large polyps including injection, endoloops, and endoclips. Many complications can actually be managed endoscopically. On the research stage, there is still a shortage of studies about many specific aspects of polypectomy, and there is a significant need for more quality studies in the future.

REFERENCES

1 **Winawer SJ**, Zauber AG, Ho MN, O'Brien MJ, Gottlieb LS, Sternberg SS, Waye JD, Schapiro M, Bond JH, Panish JF. Prevention of colorectal cancer by colonoscopic polypectomy. The National Polyp Study Workgroup. *N Engl J Med* 1993; **329**: 1977-1981

2 **Robertson DJ**. Colonoscopy for colorectal cancer prevention: is it fulfilling the promise? *Gastrointest Endosc* 2010; **71**: 118-120

3 **Waye JD**. Polyps large and small. *Gastrointest Endosc* 1992; **38**: 391-392

4 **Caputi Iambrenghi O**, Ugenti I, Martines G, Marino F, Francesco Altomare D, Memeo V. Endoscopic management of large colorectal polyps. *Int J Colorectal Dis* 2009; **24**: 749-753

5 **Khashab M**, Eid E, Rusche M, Rex DK. Incidence and predictors of "late" recurrences after endoscopic piecemeal resection of large sessile adenomas. *Gastrointest Endosc* 2009; **70**: 344-349

6 **Hayes SJ**. Assessment of colorectal adenomatous polyp size measured during pathological examination highlights the importance of accuracy. *Gastrointest Endosc* 2009; **70**: 540-541

7 **Weinberg DS**. Large adenoma recurrence after polypectomy. *Gastrointest Endosc* 2009; **70**: 350-352

8 **Mönkemüller K**, Neumann H, Malfertheiner P, Fry LC. Advanced colon polypectomy. *Clin Gastroenterol Hepatol* 2009; **7**: 641-652

9 **Singh N**, Harrison M, Rex DK. A survey of colonoscopic polypectomy practices among clinical gastroenterologists. *Gastrointest Endosc* 2004; **60**: 414-418

10 **Rex DK**. Preventing colorectal cancer and cancer mortality with colonoscopy: what we know and what we don't know. *Endoscopy* 2010; **42**: 320-323

11 **Tolliver KA**, Rex DK. Colonoscopic polypectomy. *Gastroenterol Clin North Am* 2008; **37**: 229-251, ix

12 **Gilbert DA**, DiMarino AJ, Jensen DM, Katon R, Kimmey MB, Laine LA, MacFadyen BV, Michaletz-Onody PA, Zuckerman G. Status evaluation: hot biopsy forceps. American Society for Gastrointestinal Endoscopy. Technology Assessment Committee. *Gastrointest Endosc* 1992; **38**: 753-756

13 **Williams CB**. Small polyps: the virtues and the dangers of hot biopsy. *Gastrointest Endosc* 1991; **37**: 394-395

14 **Ellis K**, Schiele M, Marquis S, Katon R. Efficacy of hot biopsy forceps. Cold micro-snare and micro-snare with cautery techniques in the removal of diminutive colonic polyps. *Gastrointest Endosc* 1997; **45**: AB107

15 **Peluso F**, Goldner F. Follow-up of hot biopsy forceps treatment of diminutive colonic polyps. *Gastrointest Endosc* 1991; **37**: 604-606

16 **Morris ML**, Tucker RD, Baron TH, Song LM. Electrosurgery in gastrointestinal endoscopy: principles to practice. *Am J Gastroenterol* 2009; **104**: 1563-1574

17 **Tappero G**, Gaia E, De Giuli P, Martini S, Gubetta L, Emanuelli G. Cold snare excision of small colorectal polyps. *Gastrointest Endosc* 1992; **38**: 310-313

18 **Carpenter S**, Petersen BT, Chuttani R, Croffie J, DiSario J, Liu J, Mishkin D, Shah R, Somogyi L, Tierney W, Song LM. Polyp-

ectomy devices. *Gastrointest Endosc* 2007; **65**: 741-749
19 **Van Gossum A**, Cozzoli A, Adler M, Taton G, Cremer M. Colonoscopic snare polypectomy: analysis of 1485 resections comparing two types of current. *Gastrointest Endosc* 1992; **38**: 472-475
20 **Swan MP**, Bourke MJ, Alexander S, Moss A, Williams SJ. Large refractory colonic polyps: is it time to change our practice? A prospective study of the clinical and economic impact of a tertiary referral colonic mucosal resection and polypectomy service (with videos). *Gastrointest Endosc* 2009; **70**: 1128-1136
21 **Conio M**, Repici A, Demarquay JF, Blanchi S, Dumas R, Filiberti R. EMR of large sessile colorectal polyps. *Gastrointest Endosc* 2004; **60**: 234-241
22 **Moss A**, Bourke MJ, Tran K, Godfrey C, McKay G, Chandra AP, Sharma S. Lesion isolation by circumferential submucosal incision prior to endoscopic mucosal resection (CSI-EMR) substantially improves en bloc resection rates for 40-mm colonic lesions. *Endoscopy* 2010; **42**: 400-404
23 **Sakamoto N**, Osada T, Shibuya T, Beppu K, Matsumoto K, Mori H, Kawabe M, Nagahara A, Otaka M, Ogihara T, Watanabe S. Endoscopic submucosal dissection of large colorectal tumors by using a novel spring-action S-O clip for traction (with video). *Gastrointest Endosc* 2009; **69**: 1370-1374
24 **Binmoeller KF**, Bohnacker S, Seifert H, Thonke F, Valdeyar H, Soehendra N. Endoscopic snare excision of "giant" colorectal polyps. *Gastrointest Endosc* 1996; **43**: 183-188
25 **Kantsevoy SV**, Adler DG, Conway JD, Diehl DL, Farraye FA, Kwon R, Mamula P, Rodriguez S, Shah RJ, Wong Kee Song LM, Tierney WM. Endoscopic mucosal resection and endoscopic submucosal dissection. *Gastrointest Endosc* 2008; **68**: 11-18
26 **Iishi H**, Tatsuta M, Iseki K, Narahara H, Uedo N, Sakai N, Ishikawa H, Otani T, Ishiguro S. Endoscopic piecemeal resection with submucosal saline injection of large sessile colorectal polyps. *Gastrointest Endosc* 2000; **51**: 697-700
27 **Zlatanic J**, Waye JD, Kim PS, Baiocco PJ, Gleim GW. Large sessile colonic adenomas: use of argon plasma coagulator to supplement piecemeal snare polypectomy. *Gastrointest Endosc* 1999; **49**: 731-735
28 **Deenadayalu VP**, Rex DK. Colon polyp retrieval after cold snaring. *Gastrointest Endosc* 2005; **62**: 253-256
29 **Waye J**. It ain't over 'til it's over: retrieval of polyps after colonoscopic polypectomy. *Gastrointest Endosc* 2005; **62**: 257-259
30 **Tierney WM**, Adler DG, Conway JD, Diehl DL, Farraye FA, Kantsevoy SV, Kaul V, Kethu SR, Kwon RS, Mamula P, Pedrosa MC, Rodriguez SA. Overtube use in gastrointestinal endoscopy. *Gastrointest Endosc* 2009; **70**: 828-834
31 **Valentine JF**. Double-channel endoscopic polypectomy technique for the removal of large pedunculated polyps. *Gastrointest Endosc* 1998; **48**: 314-316
32 **Waye JD**. Techniques for polypectomy and the problem polyp. *Tech Gastrointest Endosc* 2003; **5**: 160-165
33 **Friedland S**. Optimizing resection of difficult colon polyps. *Gastrointest Endosc* 2006; **63**: 148-149
34 **Kanamori T**, Itoh M, Yokoyama Y, Tsuchida K. Injection-incision--assisted snare resection of large sessile colorectal polyps. *Gastrointest Endosc* 1996; **43**: 189-195
35 **Ajmere N**, Barnard G, Levey J, Cave D, Demetrius L, Bhattacharya K. A Retrospective Analysis of 76 Large Polypectomies Using a Methylene Blue/Epinephrine/Saline Lift Technique with Surgical Backup. *Gastrointest Endosc* 2007; **65**: AB260
36 **Conway JD**, Adler DG, Diehl DL, Farraye FA, Kantsevoy SV, Kaul V, Kethu SR, Kwon RS, Mamula P, Rodriguez SA, Tierney WM. Endoscopic hemostatic devices. *Gastrointest Endosc* 2009; **69**: 987-996
37 **Dobrowolski S**, Dobosz M, Babicki A, Dymecki D, Hać S. Prophylactic submucosal saline-adrenaline injection in colonoscopic polypectomy: prospective randomized study. *Surg Endosc* 2004; **18**: 990-993
38 **Iishi H**, Tatsuta M, Narahara H, Iseki K, Sakai N. Endoscopic resection of large pedunculated colorectal polyps using a detachable snare. *Gastrointest Endosc* 1996; **44**: 594-597
39 **Kouklakis G**, Mpoumponaris A, Gatopoulou A, Efraimidou E, Manolas K, Lirantzopoulos N. Endoscopic resection of large pedunculated colonic polyps and risk of postpolypectomy bleeding with adrenaline injection versus endoloop and hemoclip: a prospective, randomized study. *Surg Endosc* 2009; Epub ahead of print
40 **Di Giorgio P**, De Luca L, Calcagno G, Rivellini G, Mandato M, De Luca B. Detachable snare versus epinephrine injection in the prevention of postpolypectomy bleeding: a randomized and controlled study. *Endoscopy* 2004; **36**: 860-863
41 **Paspatis GA**, Paraskeva K, Theodoropoulou A, Mathou N, Vardas E, Oustamanolakis P, Chlouverakis G, Karagiannis I. A prospective, randomized comparison of adrenaline injection in combination with detachable snare versus adrenaline injection alone in the prevention of postpolypectomy bleeding in large colonic polyps. *Am J Gastroenterol* 2006; **101**: 2805; quiz 2913
42 **Matsushita M**, Hajiro K, Takakuwa H, Kusumi F, Maruo T, Ohana M, Tominaga M, Okano A, Yunoki Y. Ineffective use of a detachable snare for colonoscopic polypectomy of large polyps. *Gastrointest Endosc* 1998; **47**: 496-499
43 **Salomon P**, Berner JS, Waye JD. Endoscopic India ink injection: a method for preparation, sterilization, and administration. *Gastrointest Endosc* 1993; **39**: 803-805
44 **Shatz BA**, Thavorides V. Colonic tattoo for follow-up of endoscopic sessile polypectomy. *Gastrointest Endosc* 1991; **37**: 59-60
45 **Lightdale CJ**. India ink colonic tattoo: blots on the record. *Gastrointest Endosc* 1991; **37**: 99-100
46 **Ginsberg GG**, Barkun AN, Bosco JJ, Burdick JS, Isenberg GA, Nakao NL, Petersen BT, Silverman WB, Slivka A, Kelsey PB. Endoscopic tattooing: February 2002. *Gastrointest Endosc* 2002; **55**: 811-814
47 **Hellmig S**, Stüber E, Kiehne K, Kosmahl M, Fölsch U. What do we really know about the long-term safety of colonic tattooing? *Gastrointest Endosc* 2005; **61**: 186-187
48 **Sawaki A**, Nakamura T, Suzuki T, Hara K, Kato T, Kato T, Hirai T, Kanemitsu Y, Okubo K, Tanaka K, Moriyama I, Kawai H, Katsurahara M, Matsumoto K, Yamao K. A two-step method for marking polypectomy sites in the colon and rectum. *Gastrointest Endosc* 2003; **57**: 735-737
49 **Raju GS**. What is new in tattooing? Custom tattooing. *Gastrointest Endosc* 2004; **59**: 328-329; author reply 329
50 **Matsushita M**, Takakuwa H, Matsubayashi Y. Effective endoscopic tattooing technique. *Gastrointest Endosc* 2004; **60**: 165-166
51 **Hixson LJ**, Fennerty MB, Sampliner RE, Garewal HS. Prospective blinded trial of the colonoscopic miss-rate of large colorectal polyps. *Gastrointest Endosc* 1991; **37**: 125-127
52 **Pohl J**, Ell C. Impact of virtual chromoendoscopy at colonoscopy: the final requiem for conventional histopathology? *Gastrointest Endosc* 2009; **69**: 723-725
53 **Buchner AM**, Shahid MW, Heckman MG, McNeil RB, Cleveland P, Gill KR, Schore A, Ghabril M, Raimondo M, Gross SA, Wallace MB. High-definition colonoscopy detects colorectal polyps at a higher rate than standard white-light colonoscopy. *Clin Gastroenterol Hepatol* 2010; **8**: 364-370
54 **Wada Y**, Kudo SE, Kashida H, Ikehara N, Inoue H, Yamamura F, Ohtsuka K, Hamatani S. Diagnosis of colorectal lesions with the magnifying narrow-band imaging system. *Gastrointest Endosc* 2009; **70**: 522-531
55 **Kanao H**, Tanaka S, Oka S, Hirata M, Yoshida S, Chayama K. Narrow-band imaging magnification predicts the histology and invasion depth of colorectal tumors. *Gastrointest Endosc* 2009; **69**: 631-636
56 **Rastogi A**, Bansal A, Wani S, Callahan P, McGregor DH, Cherian R, Sharma P. Narrow-band imaging colonoscopy--a

pilot feasibility study for the detection of polyps and correlation of surface patterns with polyp histologic diagnosis. *Gastrointest Endosc* 2008; **67**: 280-286
57 **Buchner AM**, Wallace MB. Novel endoscopic approaches in detecting colorectal neoplasia: macroscopes, microscopes, and metal detectors. *Gastroenterology* 2008; **135**: 1035-1037
58 **Buchner AM**, Shahid MW, Heckman MG, Krishna M, Ghabril M, Hasan M, Crook JE, Gomez V, Raimondo M, Woodward T, Wolfsen HC, Wallace MB. Comparison of probe-based confocal laser endomicroscopy with virtual chromoendoscopy for classification of colon polyps. *Gastroenterology* 2010; **138**: 834-842
59 **Gómez V**, Buchner AM, Dekker E, van den Broek FJ, Meining A, Shahid MW, Ghabril MS, Fockens P, Heckman MG, Wallace MB. Interobserver agreement and accuracy among international experts with probe-based confocal laser endomicroscopy in predicting colorectal neoplasia. *Endoscopy* 2010; **42**: 286-291
60 **Dobrowolski S**, Dobosz M, Babicki A, Głowacki J, Nałecz A. Blood supply of colorectal polyps correlates with risk of bleeding after colonoscopic polypectomy. *Gastrointest Endosc* 2006; **63**: 1004-1009
61 **Watabe H**, Yamaji Y, Okamoto M, Kondo S, Ohta M, Ikenoue T, Kato J, Togo G, Matsumura M, Yoshida H, Kawabe T, Omata M. Risk assessment for delayed hemorrhagic complication of colonic polypectomy: polyp-related factors and patient-related factors. *Gastrointest Endosc* 2006; **64**: 73-78
62 **Raju GS**, Gajula L. Endoclips for GI endoscopy. *Gastrointest Endosc* 2004; **59**: 267-279
63 **Sobrino-Faya M**, Martínez S, Gómez Balado M, Lorenzo A, Iglesias-García J, Iglesias-Canle J, Domínquez Muñoz JE. Clips for the prevention and treatment of postpolypectomy bleeding (hemoclips in polypectomy). *Rev Esp Enferm Dig* 2002; **94**: 457-462
64 **Friedland S**, Sedehi D, Soetikno R. Colonoscopic polypectomy in anticoagulated patients. *World J Gastroenterol* 2009; **15**: 1973-1976
65 **Singh H**, Penfold RB, DeCoster C, Kaita L, Proulx C, Taylor G, Bernstein CN, Moffatt M. Colonoscopy and its complications across a Canadian regional health authority. *Gastrointest Endosc* 2009; **69**: 665-671
66 **Levin TR**, Zhao W, Conell C, Seeff LC, Manninen DL, Shapiro JA, Schulman J. Complications of colonoscopy in an integrated health care delivery system. *Ann Intern Med* 2006; **145**: 880-886
67 **Pochapin MB**. Understanding the risks of colonoscopy: looking forward. *Gastrointest Endosc* 2009; **69**: 672-674
68 **Arora G**, Mannalithara A, Singh G, Gerson LB, Triadafilopoulos G. Risk of perforation from a colonoscopy in adults: a large population-based study. *Gastrointest Endosc* 2009; **69**: 654-664
69 **Raju GS**, Fritscher-Ravens A, Rothstein RI, Swain P, Gelrud A, Ahmed I, Gomez G, Winny M, Sonnanstine T, Bergström M, Park PO. Endoscopic closure of colon perforation compared to surgery in a porcine model: a randomized controlled trial (with videos). *Gastrointest Endosc* 2008; **68**: 324-332

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GUIDELINES FOR BASIC SCIENCE

Interleukin-21 triggers effector cell responses in the gut

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Abstract

In the gut of patients with Crohn's disease and patients with ulcerative colitis, the major forms of inflammatory bowel diseases (IBD) in humans, the tissue-damaging immune response is mediated by an active cross-talk between immune and non-immune cells. Accumulating evidence indicates also that cytokines produced by these cells play a major role in initiating and shaping this pathologic process. One such cytokine seems to be interleukin (IL)-21, a member of the common γ-chain-receptor family. IL-21 is produced in excess in the inflamed intestine of patients with IBD mostly by activated CD4+ T helper cells co-expressing interferon-γ and follicular T helper cells. Moreover, both *in vitro* and *in vivo* studies indicate that excessive IL-21 production leads to the activation of multiple signaling pathways that expand and sustain the ongoing mucosal inflammation. In this article, we review the available data supporting the pathogenic role of IL-21 in IBD.

Key words: Interleukin-21; Gut; T cells; Epithelial cells; Fibroblasts

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INTRODUCTION

The organized lymphoid tissue of the gastrointestinal tract contains large numbers of immune cells that are deputed both to protect from infectious diseases and to evoke immune tolerance[1,2]. Perturbations in this delicately balanced microenvironment can promote the collapse of tolerance, thus leading to chronic inflammation that alters the integrity and function of the gut[3]. This occurs for example in patients with Crohn's disease (CD) and patients with ulcerative colitis (UC), the major forms of inflammatory bowel diseases (IBD) in humans[4]. In both these conditions, the tissue damage is mediated by an excessive and poorly controlled immune-inflammatory reaction directed against components of the normal bacterial flora[5,6]. Evidence also indicates that an active and dynamic interplay between immune and non-immune cells plays a major role in initiating and shaping this pathologic process, and that cytokines are essential mediators of this cross-talk[7-9]. One such cytokine seems to be interleukin (IL)-21, a product of activated CD4+ T helper (Th) cells, follicular Th cells (TFH), and Natural killer (NK) T cells, which exerts regulatory effects on multiple cell types[10-13]. In this article, we review the available data supporting the pathogenic role of IL-21 in IBD.

IL-21 IS MADE BY TH1 CELLS AND FOLLICULAR TH CELLS IN THE HUMAN GUT

Initial studies conducted in our laboratory showed that IL-21 protein is over-produced in the inflamed gut of

patients with CD and patients with UC as compared to normal controls[14]. These data were confirmed by a recent study showing that IL-21 mRNA expression is increased in rectal mucosa from patients with active UC compared to UC patients in remission and healthy controls and that, in UC, IL-21 gene expression correlates with histological activity of the disease[15]. A genome-wide association study for IBD has identified risk variants in the chromosomal 4q27 region harbouring the *IL-2* and *IL-21* genes, suggesting that polymorphism(s) in this region might contribute to regulation of IL-21 production/function[16-18]. However, it is noteworthy that expression of IL-21 in the uninflamed mucosa of IBD patients does not differ from that seen in the normal colonic mucosa, and that peripheral blood T cells isolated from IBD patients and healthy controls express similar levels of IL-21[14]. Therefore, it is plausible that up-regulation of IL-21 in IBD is strictly linked to the ongoing mucosal inflammation.

In both CD and UC, IL-21 is made by CD4+ but not CD8+ T cells[14]. By flow-cytometry it was also shown that the majority of IL-21-producing CD4+ T-LPL co-express interferon (IFN)-γ, and to a lesser extent IL-17A, supporting the hypothesis that, in IBD, IL-21 is preferentially made by Th1 rather than Th17 cells[19]. At the present time, it remains unclear how IL-21-positive cells co-expressing IFN-γ differentiate in the human gut. Since Th1 cells are abundant in the human gut, and particularly in CD mucosa[20-22], it is conceivable that Th1 cells can acquire the ability to make IL-21 in response to specific stimuli. Indeed, we have recently shown that *in vitro* stimulation of intestinal lamina propria (LP) CD4+ T cells with IL-12, the major inducer of Th1 cell response, enhances the fraction of cells producing IL-21 or both IL-21 and IFN-γ[19].

IL-21 is also produced by TFH cells in the human gut, and the fraction of IL-21-producing TFH cells is significantly higher in CD than in UC and controls[19]. Interestingly, activation of mucosal T cells with IL-12 leads to enhanced production of IL-21 by TFH cells[19], thus confirming that IL-12-driven signals positively regulate IL-21 production in the gut.

IL-21 ENHANCES INFLAMMATORY PATHWAYS IN THE GUT

A large body of evidence supports the concept that excessive production of IL-21 in the gut has deleterious consequences for the host. IL-21 is highly produced in the gut of wild-type mice with dextran sulfate sodium (DSS)- and trinitrobenzene sulfonic acid-relapsing (TNBS)-induced colitis[23]. Notably, IL-21-deficient mice are largely protected against disease in both models[23]. Amelioration of both DSS- and TNBS-induced colitis in IL-21-knockout mice is associated with a marked decrease in Th17-related molecules, such as IL-17 and IL-17F. Administration of IL-21R/Fc, a fusion protein that binds to IL-21 and prevents it activating cell-surface receptors, in wild-type mice attenuates DSS-colitis, confirming the pro-inflammatory role of IL-21 in this model[23]. A similar scenario emerges from studies in human IBD[19]. Stimulation of intestinal mucosal T cells with IL-21 results in enhanced activation of transcription factors (i.e. Stat3, Stat4 and T-bet) and marked synthesis of IFN-γ and IL-21 itself[14]. Moreover, treatment of CD mucosal cells with IL-21R/Fc reduces Stat4 and T-bet and inhibits IFN-γ production. Neutralization of IL-21 in CD mucosal cell cultures leads also to a decreased expression of IL-17A[23]. Taken together these data indicate that IL-21 is able to expand Th1 and Th17 cell responses in the gut, even though further experimentation is needed to elucidate the basic mechanism by which IL-21 exerts these regulatory effects.

Initially described as an important regulator of the function of immune cells[24,25], IL-21 has been recently shown to also regulate the activity of non-immune cells. Gut myofibroblasts and epithelial cells express constitutively IL-21R and are able to respond to IL-21[26]. In particular, stimulation of colonic myofibroblasts with IL-21 enhances the synthesis of matrix metalloproteinases (MMPs)[26], a family of proteases that are supposed to participate in the tissue damage and remodelling occurring in IBD[27,28]. The IL-21-driven induction of MMPs can be potentiated by tumor necrosis factor α, and associates with no change in the production of tissue inhibitors of MMPs[26]. Regulation of MMPs by IL-21 does not however seem to occur at the transcriptional level, because stimulation of fibroblasts with IL-21 does not alter the MMP RNA expression[26]. Additionally, the intracellular level of MMP proteins is not increased by IL-21, and the IL-21-induced MMP synthesis is not affected by inhibitors of gene transcription and *de novo* protein synthesis[26]. Therefore, it is plausible that IL-21 preferentially increases the secretion of either pre-constituted or newly synthesized MMPs. The *in vivo* relevance of these findings relates to the demonstration that supernatants of CD mucosal cells induce myofibroblasts to secrete MMP and this is partially inhibitable by IL-21R/Fc[26].

IL-21 induces activation of mitogen activated protein kinases in colonic epithelial cells thereby promoting the secretion of macrophage inflammatory protein (MIP)-3α[29], a chemokine up-regulated on the inflamed gut epithelium of IBD patients and involved in the recruitment of T cells in the gut mucosa[30,31]. In line with these observations, blockade of endogenous IL-21 in cultures of IBD mucosal explants reduces MIP-3α synthesis by epithelial cells[29].

IL-21 INHIBITS REGULATORY T CELL DIFFERENTIATION AND MAKES CD4+ T CELLS RESISTANT TO TREGS-MEDIATED IMMUNE-SUPPRESSION

Regulatory T cells (Tregs) play an important role in maintaining homeostasis and preventing autoimmunity in various organs, including the gut[32,33]. Tregs specifically express the transcription factor forkhead winged helix transcription factor gene (Foxp3), which is also functionally required for their regulatory activity[32]. In addition to naturally occur-

ring Tregs that are produced by the thymus as a functionally distinct and mature population of T cells[33], Tregs can arise in the periphery upon conversion of CD4+CD25- T cells into Foxp3-positive-CD4+CD25+ cells in response to activating stimuli and transforming growth factor (TGF)-β1[34,35]. This phenomenon seems to occur in the normal gut, where TGF-β1 synergizes with other regulatory molecules (e.g. IL-10, retinoic acid) in mounting an effective counter-regulatory response[36,37]. However, if activation of naïve CD4+CD25- T cells occurs in the presence of TGF-β1 and inflammatory stimuli, they tend to differentiate into effector Th17 cells rather than into Tregs[38]. IL-21 seems to accomplish this function, given that it can cross-regulate Tregs induction and direct the development of Th17 cells[39]. Interestingly, colitis induced by the transfer of naïve T cells into severe combined immunodeficient mice is suppressed by TGF-β1-induced Tregs generated *in vitro* in the absence of IL-21 but not by T cells generated in the presence of TGF-β1 and IL-21[40]. By contrast, these latter T cells exacerbate colitis with increased expression of IL-17 and a reduced number of Foxp3-expressing cells in the gut mucosa[40]. Consistent with this is the demonstration that blockade of IL-21 associates with high numbers of Foxp3-positive Tregs and reduced tissue damage in the colon and small intestine of mice with acute graft *vs* host disease[41].

IL-21 is also able to counteract the regulatory effects of Tregs by providing human CD4+ T cells signals that raise their threshold for suppression by Tregs[42]. Collectively these observations delineate another mechanism by which IL-21 contributes to amplify the ongoing inflammation in IBD.

CONCLUSION

There is no doubt that IL-21 modulates the activity of several cell types that orchestrate the tissue damage in IBD, and that blockade of IL-21 signalling attenuates the ongoing mucosal inflammation in experimental models of IBD. Therefore, it is conceivable that the near future will witness the use of novel therapeutic strategies aimed at inhibiting IL-21 activity in IBD. However, some important issues related to the blockade of IL-21 function must be taken into consideration before moving into the clinic. For instance, we should not forget that IL-21 plays a decisive role in the control of B cell and plasma cell function, and that IL-21 signalling may attenuate the course of IgE-mediated diseases[24,25]. Moreover, blockade of IL-21 could potentially enhance the risk of malignancies and exacerbate chronic viral infections given the ability of IL-21 to trigger CD8+ T cell-dependent immune reactions against tumors and viruses[43-48]. At least in some circumstances, IL-21 stimulates IL-10 production, thereby promoting tolerogenic rather than inflammatory responses. If so, anti-IL-21 therapy could paradoxically enhance the risk of autoimmunity.

REFERENCES

1 **Mowat AM**, Parker LA, Beacock-Sharp H, Millington OR, Chirdo F. Oral tolerance: overview and historical perspectives. *Ann N Y Acad Sci* 2004; **1029**: 1-8
2 **Mowat AM**. Dendritic cells and immune responses to orally administered antigens. *Vaccine* 2005; **23**: 1797-1799
3 **Macdonald TT**, Monteleone G. Immunity, inflammation, and allergy in the gut. *Science* 2005; **307**: 1920-1925
4 **Podolsky DK**. Inflammatory bowel disease. *N Engl J Med* 2002; **347**: 417-429
5 **Strober W**, Fuss I, Mannon P. The fundamental basis of inflammatory bowel disease. *J Clin Invest* 2007; **117**: 514-521
6 **Abraham C**, Cho JH. Inflammatory bowel disease. *N Engl J Med* 2009; **361**: 2066-2078
7 **Elson CO**, Cong Y, McCracken VJ, Dimmitt RA, Lorenz RG, Weaver CT. Experimental models of inflammatory bowel disease reveal innate, adaptive, and regulatory mechanisms of host dialogue with the microbiota. *Immunol Rev* 2005; **206**: 260-276
8 **MacDonald TT**, Bajaj-Elliott M, Pender SL. T cells orchestrate intestinal mucosal shape and integrity. *Immunol Today* 1999; **20**: 505-510
9 **Fantini MC**, Monteleone G, Macdonald TT. New players in the cytokine orchestra of inflammatory bowel disease. *Inflamm Bowel Dis* 2007; **13**: 1419-1423
10 **Parrish-Novak J**, Dillon SR, Nelson A, Hammond A, Sprecher C, Gross JA, Johnston J, Madden K, Xu W, West J, Schrader S, Burkhead S, Heipel M, Brandt C, Kuijper JL, Kramer J, Conklin D, Presnell SR, Berry J, Shiota F, Bort S, Hambly K, Mudri S, Clegg C, Moore M, Grant FJ, Lofton-Day C, Gilbert T, Rayond F, Ching A, Yao L, Smith D, Webster P, Whitmore T, Maurer M, Kaushansky K, Holly RD, Foster D. Interleukin 21 and its receptor are involved in NK cell expansion and regulation of lymphocyte function. *Nature* 2000; **408**: 57-63
11 **Nurieva R**, Yang XO, Martinez G, Zhang Y, Panopoulos AD, Ma L, Schluns K, Tian Q, Watowich SS, Jetten AM, Dong C. Essential autocrine regulation by IL-21 in the generation of inflammatory T cells. *Nature* 2007; **448**: 480-483
12 **Vogelzang A**, McGuire HM, Yu D, Sprent J, Mackay CR, King C. A fundamental role for interleukin-21 in the generation of T follicular helper cells. *Immunity* 2008; **29**: 127-137
13 **Coquet JM**, Kyparissoudis K, Pellicci DG, Besra G, Berzins SP, Smyth MJ, Godfrey DI. IL-21 is produced by NKT cells and modulates NKT cell activation and cytokine production. *J Immunol* 2007; **178**: 2827-2834
14 **Monteleone G**, Monteleone I, Fina D, Vavassori P, Del Vecchio Blanco G, Caruso R, Tersigni R, Alessandroni L, Biancone L, Naccari GC, MacDonald TT, Pallone F. Interleukin-21 enhances T-helper cell type I signaling and interferon-gamma production in Crohn's disease. *Gastroenterology* 2005; **128**: 687-694
15 **Yamamoto-Furusho JK**, Miranda-Pérez E, Fonseca-Camarillo G, Sánchez-Muñoz F, Barreto-Zuñiga R, Dominguez-Lopez A. Interleukin 21 expression is increased in rectal biopsies from patients with ulcerative colitis. *Inflamm Bowel Dis* 2010; **16**: 1090
16 **Márquez A**, Orozco G, Martínez A, Palomino-Morales R, Fernández-Arquero M, Mendoza JL, Taxonera C, Díaz-Rubio M, Gómez-García M, Nieto A, López-Nevot MA, de la Concha EG, Martín J, Urcelay E. Novel association of the interleukin 2-interleukin 21 region with inflammatory bowel disease. *Am J Gastroenterol* 2009; **104**: 1968-1975
17 **van Heel DA**, Franke L, Hunt KA, Gwilliam R, Zhernakova A, Inouye M, Wapenaar MC, Barnardo MC, Bethel G, Holmes GK, Feighery C, Jewell D, Kelleher D, Kumar P, Travis S, Walters JR, Sanders DS, Howdle P, Swift J, Playford RJ, McLaren WM, Mearin ML, Mulder CJ, McManus R, McGinnis R, Cardon LR, Deloukas P, Wijmenga C. A genome-wide association study for celiac disease identifies risk variants in the region harboring IL2 and IL21. *Nat Genet* 2007; **39**: 827-829
18 **Festen EA**, Goyette P, Scott R, Annese V, Zhernakova A, Lian J, Lefèbvre C, Brant SR, Cho JH, Silverberg MS, Taylor KD, de Jong DJ, Stokkers PC, Mcgovern D, Palmieri O, Achkar JP, Xavier RJ, Daly MJ, Duerr RH, Wijmenga C, Weersma RK,

Rioux JD. Genetic variants in the region harbouring IL2/IL21 associated with ulcerative colitis. *Gut* 2009; **58**: 799-804
19 **Sarra M**, Monteleone I, Stolfi C, Fantini MC, Sileri P, Sica G, Tersigni R, Macdonald TT, Pallone F, Monteleone G. Interferon-gamma-expressing cells are a major source of interleukin-21 in inflammatory bowel diseases. *Inflamm Bowel Dis* 2010; **16**: 1332-1339
20 **Fuss IJ**, Neurath M, Boirivant M, Klein JS, de la Motte C, Strong SA, Fiocchi C, Strober W. Disparate CD4+ lamina propria (LP) lymphokine secretion profiles in inflammatory bowel disease. Crohn's disease LP cells manifest increased secretion of IFN-gamma, whereas ulcerative colitis LP cells manifest increased secretion of IL-5. *J Immunol* 1996; **157**: 1261-1270
21 **Neurath MF**, Weigmann B, Finotto S, Glickman J, Nieuwenhuis E, Iijima H, Mizoguchi A, Mizoguchi E, Mudter J, Galle PR, Bhan A, Autschbach F, Sullivan BM, Szabo SJ, Glimcher LH, Blumberg RS. The transcription factor T-bet regulates mucosal T cell activation in experimental colitis and Crohn's disease. *J Exp Med* 2002; **195**: 1129-1143
22 **Monteleone G**, Biancone L, Marasco R, Morrone G, Marasco O, Luzza F, Pallone F. Interleukin 12 is expressed and actively released by Crohn's disease intestinal lamina propria mononuclear cells. *Gastroenterology* 1997; **112**: 1169-1178
23 **Fina D**, Sarra M, Fantini MC, Rizzo A, Caruso R, Caprioli F, Stolfi C, Cardolini I, Dottori M, Boirivant M, Pallone F, Macdonald TT, Monteleone G. Regulation of gut inflammation and th17 cell response by interleukin-21. *Gastroenterology* 2008; **134**: 1038-1048
24 **Monteleone G**, Pallone F, Macdonald TT. Interleukin-21 (IL-21)-mediated pathways in T cell-mediated disease. *Cytokine Growth Factor Rev* 2009; **20**: 185-191
25 **Spolski R**, Leonard WJ. IL-21 and T follicular helper cells. *Int Immunol* 2010; **22**: 7-12
26 **Monteleone G**, Caruso R, Fina D, Peluso I, Gioia V, Stolfi C, Fantini MC, Caprioli F, Tersigni R, Alessandroni L, MacDonald TT, Pallone F. Control of matrix metalloproteinase production in human intestinal fibroblasts by interleukin 21. *Gut* 2006; **55**: 1774-1780
27 **Kruidenier L**, MacDonald TT, Collins JE, Pender SL, Sanderson IR. Myofibroblast matrix metalloproteinases activate the neutrophil chemoattractant CXCL7 from intestinal epithelial cells. *Gastroenterology* 2006; **130**: 127-136
28 **Pender SL**, Fell JM, Chamow SM, Ashkenazi A, MacDonald TT. A p55 TNF receptor immunoadhesin prevents T cell-mediated intestinal injury by inhibiting matrix metalloproteinase production. *J Immunol* 1998; **160**: 4098-4103
29 **Caruso R**, Fina D, Peluso I, Stolfi C, Fantini MC, Gioia V, Caprioli F, Del Vecchio Blanco G, Paoluzi OA, Macdonald TT, Pallone F, Monteleone G. A functional role for interleukin-21 in promoting the synthesis of the T-cell chemoattractant, MIP-3alpha, by gut epithelial cells. *Gastroenterology* 2007; **132**: 166-175
30 **Kwon JH**, Keates S, Bassani L, Mayer LF, Keates AC. Colonic epithelial cells are a major site of macrophage inflammatory protein 3alpha (MIP-3alpha) production in normal colon and inflammatory bowel disease. *Gut* 2002; **51**: 818-826
31 **Teramoto K**, Miura S, Tsuzuki Y, Hokari R, Watanabe C, Inamura T, Ogawa T, Hosoe N, Nagata H, Ishii H, Hibi T. Increased lymphocyte trafficking to colonic microvessels is dependent on MAdCAM-1 and C-C chemokine mLARC/CCL20 in DSS-induced mice colitis. *Clin Exp Immunol* 2005; **139**: 421-428
32 **Hori S**, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 2003; **299**: 1057-1061
33 **Sakaguchi S**, Ono M, Setoguchi R, Yagi H, Hori S, Fehervari Z, Shimizu J, Takahashi T, Nomura T. Foxp3+ CD25+ CD4+ natural regulatory T cells in dominant self-tolerance and autoimmune disease. *Immunol Rev* 2006; **212**: 8-27
34 **Fantini MC**, Becker C, Monteleone G, Pallone F, Galle PR, Neurath MF. Cutting edge: TGF-beta induces a regulatory phenotype in CD4+CD25- T cells through Foxp3 induction and down-regulation of Smad7. *J Immunol* 2004; **172**: 5149-5153
35 **Chen W**, Jin W, Hardegen N, Lei KJ, Li L, Marinos N, McGrady G, Wahl SM. Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J Exp Med* 2003; **198**: 1875-1886
36 **Coombes JL**, Siddiqui KR, Arancibia-Cárcamo CV, Hall J, Sun CM, Belkaid Y, Powrie F. A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. *J Exp Med* 2007; **204**: 1757-1764
37 **El-Asady R**, Yuan R, Liu K, Wang D, Gress RE, Lucas PJ, Drachenberg CB, Hadley GA. TGF-{beta}-dependent CD103 expression by CD8(+) T cells promotes selective destruction of the host intestinal epithelium during graft-versus-host disease. *J Exp Med* 2005; **201**: 1647-1657
38 **Mucida D**, Park Y, Kim G, Turovskaya O, Scott I, Kronenberg M, Cheroutre H. Reciprocal TH17 and regulatory T cell differentiation mediated by retinoic acid. *Science* 2007; **317**: 256-260
39 **Yang L**, Anderson DE, Baecher-Allan C, Hastings WD, Bettelli E, Oukka M, Kuchroo VK, Hafler DA. IL-21 and TGF-beta are required for differentiation of human T(H)17 cells. *Nature* 2008; **454**: 350-352
40 **Fantini MC**, Rizzo A, Fina D, Caruso R, Becker C, Neurath MF, Macdonald TT, Pallone F, Monteleone G. IL-21 regulates experimental colitis by modulating the balance between Treg and Th17 cells. *Eur J Immunol* 2007; **37**: 3155-3163
41 **Bucher C**, Koch L, Vogtenhuber C, Goren E, Munger M, Panoskaltsis-Mortari A, Sivakumar P, Blazar BR. IL-21 blockade reduces graft-versus-host disease mortality by supporting inducible T regulatory cell generation. *Blood* 2009; **114**: 5375-5384
42 **Peluso I**, Fantini MC, Fina D, Caruso R, Boirivant M, MacDonald TT, Pallone F, Monteleone G. IL-21 counteracts the regulatory T cell-mediated suppression of human CD4+ T lymphocytes. *J Immunol* 2007; **178**: 732-739
43 **Fröhlich A**, Kisielow J, Schmitz I, Freigang S, Shamshiev AT, Weber J, Marsland BJ, Oxenius A, Kopf M. IL-21R on T cells is critical for sustained functionality and control of chronic viral infection. *Science* 2009; **324**: 1576-1580
44 **Yi JS**, Du M, Zajac AJ. A vital role for interleukin-21 in the control of a chronic viral infection. *Science* 2009; **324**: 1572-1576
45 **Elsaesser H**, Sauer K, Brooks DG. IL-21 is required to control chronic viral infection. *Science* 2009; **324**: 1569-1572
46 **Andorsky DJ**, Timmerman JM. Interleukin-21: biology and application to cancer therapy. *Expert Opin Biol Ther* 2008; **8**: 1295-1307
47 **Spolski R**, Leonard WJ. The Yin and Yang of interleukin-21 in allergy, autoimmunity and cancer. *Curr Opin Immunol* 2008; **20**: 295-301
48 **Skak K**, Kragh M, Hausman D, Smyth MJ, Sivakumar PV. Interleukin 21: combination strategies for cancer therapy. *Nat Rev Drug Discov* 2008; **7**: 231-240

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ORIGINAL ARTICLE

Non-peptidyl low molecular weight radical scavenger IAC attenuates DSS-induced colitis in rats

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Abstract

AIM: To investigate the effects of the free radical scavenger bis(1-hydroxy-2,2,6,6-tetramethyl-4-piperidinyl) decandioate (IAC) in the dextran sodium sulphate (DSS) experimental model of ulcerative colitis.

METHODS: Colitis was induced in Sprague Dawley male rats by administration of 5% DSS in drinking water. IAC (30 mg/kg, lipophilic or hydrophilic form) was administered daily (orally or ip) for 6 d until sacrifice. Colonic damage was assessed by means of indirect (Disease Activity Index score) and direct measures (macroscopic and microscopic scores) and myeloperoxidase (MPO) activity. Neutrophil infiltration within the tissue and glutathione S-transferase activity were also investigated.

RESULTS: DSS-induced colitis impaired body weight gain and markedly increased all inflammatory parameters. Six-day treatment with lipophilic IAC significantly reduced intestinal damage caused by inflammation, induced a down-regulation in MPO activity (0.72 ± 0.12 and 0.45 ± 0.12 with lipophilic IAC *po* and ip, respectively, *vs* 1.10 ± 0.27 in untreated DSS colitis animals) and minimized DSS-induced neutrophil infiltration, while hydrophilic IAC administered orally did not ameliorate DSS-induced damage.

CONCLUSION: These results support the hypothesis that reactive oxygen metabolites contribute to inflammation and that the radical scavenger IAC has therapeutic potential in inflammatory bowel disease.

Key words: Dextran sodium sulphate-induced colitis; Oxidative damage; Inflammatory bowel disease; Bis(1-hydroxy-2,2,6,6-tetramethyl-4-piperidinyl)decandioate; Radical scavenger; Animal models

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INTRODUCTION

Ulcerative colitis (UC) and Crohn's disease (CD) are chronic inflammatory diseases of the gut of unclear etiology: environmental and genetic factors regulating mucosal immune response, mucosal barrier function and response to intestinal microflora are all thought to contribute to the pathogenesis of these diseases, characterized by mucosal inflammatory infiltrates, intestinal barrier function alteration and erosive loss of mucosa and submucosa[1,2].

Because of extensive mucosal damage and massive infiltration of polymorphonuclear and mononuclear leukocytes[1,2], reactive oxygen and nitrogen radical species are produced and released, resulting in potential oxidation and peroxidation of a large number of molecules (e.g. proteins, lipids and DNA). Indeed, the intestinal mucosa of patients with inflammatory bowel disease (IBD) is characterized by radical species overproduction and imbalance of the most important antioxidants[3,4] leading to oxidative damage; self-sustaining cycles of oxidant production may amplify inflammation and mucosal injury in UC, where activated neutrophils and macrophages mainly contribute to active lesions[5,6]. The phagocytes present in the mucosa of IBD patients, indeed, can produce reactive oxygen metabolites such as superoxide and hydrogen peroxide, through both respiration burst and prostaglandin and leukotriene metabolism. Radical species released during inflammation increase mucosal permeability and contribute to the recruitment and activation of further neutrophils, thus initiating and/or propagating inflammation and tissue damage[6].

Antioxidant compounds and free radical scavengers improved colitis in several experimental models[7,8]. Recently, in a proof of concept study, we have shown that the radical scavenger bis(1-hydroxy-2,2,6,6-tetramethyl-4-piperidinyl)decandioate (IAC) protects the rat colon from DNBS-induced colitis, which mimics human CD[9]. DNBS is a contact sensitizing allergen which causes immunological activation; acute pathological features include focal necrosis and acute inflammation, followed by a chronic infiltration of mononuclear cells[10].

In this present study, we investigated the effects of IAC in a different model of colitis, i.e. one that is induced by dextran sodium sulphate (DSS), which differs from the DNBS model in terms of tissue inflammatory and immunological activation, as well as severity of the inflammatory process, and is thought to closely mimic human UC[10]. In the DSS model, as in UC, only the mucosa is affected by inflammation; early damage includes shortening and dropout of crypts, particularly over lymphoid aggregates, progressing to focal ulceration, mononuclear cell and neutrophil infiltration. Similar to UC, DSS provokes acute inflammation and macrophage activation, with consequent epithelial cell injury and activation of innate immune responses by luminal bacterial components and eventual activation of T cells[2,11,12].

We tested two different forms of IAC in DSS colitis, with either hydrophilic or lipophilic character. Due to its peculiar physico-chemical properties, IAC readily diffuses through the cellular membrane and can reach virtually any compartment where the production of free radicals occurs, possessing equal radical scavenging activity[13]. This represents an advantage, because it can directly react with free radicals.

MATERIALS AND METHODS

Animals

Male Sprague Dawley rats (180-200 g body weight; Harlan Italy, S. Pietro al Natisone, Udine, Italy) were used in this study. Animals were housed in a controlled environment and had free access to food and water throughout the study. Before starting any experimental procedure, in order to minimize stress, animals of all experimental groups were weighed and gently manipulated in the laboratory environment for 30 min every day for at least 1 wk. All experiments were carried out according to the guidelines set forth by EEC Directive 86/609 on the care and use of experimental animals. The protocol for induction of colitis was reviewed by the Institutional Committee on the care and use of experimental animals of the University of Bologna and was authorized by the Italian Ministry of Health. A persistently hunched posture and labored respiration, a markedly erect coat and a weight loss of more than 20% were considered as humane end-points at which to euthanize the animals.

Induction of colitis

Colitis was induced using a previously described method[14]. DSS (molecular weight, 40 kilodaltons; ICN Biomedicals Inc, Aurora, OH) was added to drinking water at a final concentration of 5% (wt/vol) for 5 d. Controls were all time-matched and consisted of rats receiving normal drinking water only. The DSS solution was replenished daily and mean DSS consumption was noted per cage at the end of 5-d treatment.

Experimental design

We studied groups of rats with and without colitis (n = 6-12 per group), which were treated with the radical scavenger IAC (Figure 1) synthesized in our laboratory[13], starting the day before the induction of colitis for 5 d. IAC (30 mg/kg), hydrophilic or lipophilic form, was administered once daily at the same time (orally or ip) as water solution or suspension. In our previous work, we observed that treatment with hydrophilic IAC *po* in DNBS-induced colitis induced only a minor protective effect, while when administered ip, it was unable to reduce inflammation, therefore we decided to only test the hydrophilic form *po* in this study[9].

The dose of 30 mg/kg was selected on the basis of previous studies, in which IAC showed the best antioxidant activity[15] and protective effect in DNBS-induced colitis[9]. At the end of this 7-d period, the animals were killed and their colons collected for further analysis.

Tissue collection

Before tissue samples were collected, the entire colon was

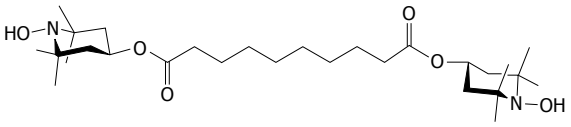

Figure 1 Bis(1-hydroxy-2,2,6,6-tetramethyl-4-piperidinyl)decandioate structure.

removed, the length starting from 1 cm above the anus to the top of the cecum and the weight of the colon still containing fecal contents were measured. Then the colon was opened longitudinally and washed with phosphate buffered saline (PBS) to remove luminal contents; at this time, the consistency of any stools found within the colon and the gross macroscopic appearance of the colon were noted. Whole-wall colonic samples were pinned flat on wax, fixed in cold neutral 4% formalin and then placed in 25% sucrose in PBS at 4℃ for cryoprotection and embedded in optimal cutting temperature tissue freezing medium. Seven-micron-thick sections of colon were cut, serially mounted on glass and processed for routine hematoxylin-eosin (HE) staining and naphthol AS-D chloroacetate esterase assay. Specimens of colonic tissue were also removed, snap frozen in liquid nitrogen and stored at -80℃ until subsequent assays.

Assessment of the severity of colitis

Disease activity index: Disease activity index (DAI) scores have historically well correlated with pathological findings in a DSS-induced model of IBD[16]. DAI is the combined score of weight loss, stool consistency and bleeding, as detailed in Table 1. All parameters were scored from day 0 to day 5 during DSS treatment.

Total macroscopic score: When rats were sacrificed, the colon was removed, opened longitudinally and washed with PBS to remove luminal contents and macroscopic damage was immediately assessed. The macroscopic parameters analyzed before tissue samples were collected were: colon length starting 1 cm above the anus to the top of the cecum, weight of the colon still containing fecal contents, stool consistency and gross macroscopic appearance of the colon. Decreases in filled colon weight are indicative of colonic hypermotility[17]. Indeed, colons from animals with severe colitis can be seen to be nearly devoid of fecal contents. Colon shrinkage is also commonly observed in DSS colitis and is indicative of colonic smooth muscle contraction[17,18]. Total macroscopic score was assigned according to a previously described scoring system[16]; details for each parameter are reported in Table 2.

Histology: Seven-micron-thick sections of colon were cut, serially mounted on glass and processed for routine HE staining. Colonic damage was scored based on a published scoring system that considers amount of inflammation (from 0, none, to 3, severe), extent of inflammation (from 0, none, to 3, transmural), crypt damage (from 0, none, to 4, entire crypt and epithelium lost) and tissue regeneration (from 4, no tissue repair, to 0, complete regeneration or normal tissue)[11].

Table 1 Disease activity index score parameters

Stool consistency	Bleeding	Weight loss	Maximum score
0 = formed	0 = normal color stool	0 = no weight loss	10
1 = mild-soft	1 = brown color stool	1 = 5%-10% weight loss	
2 = very soft	2 = reddish color stool	2 = 11%-15% weight loss	
3 = watery stool	3 = bloody stool	3 = 16%-20% weight loss	
		4 = > 20% weight loss	

Myeloperoxidase assay

Specimens of colonic tissue (50 mg) were assayed using a previously described method[19]. Myeloperoxidase (MPO) is a granule-associated enzyme present in neutrophils and other cells of myeloid origin, and widely used as a marker of intestinal inflammation. Colonic tissues were homogenized in ice-cold potassium phosphate buffer (pH 6.0) and centrifuged for 10 min at 6000 *g* at 4℃. The supernatants were then collected, added to a solution of *O*-dianisidine (Sigma-Aldrich, Milan, Italy) and hydrogen peroxide and assayed to assess MPO activity.

MPO was expressed in units per milligram of tissue, where 1 U corresponds to the activity required to degrade 1 μmol of hydrogen peroxide in 1 min at room temperature.

Glutathione S-transferase activity

Colonic samples (25 mg) were obtained using a previously described method[9]. The supernatant was collected and then assayed for glutathione S-transferase (GST) activity[20]. Assessment of protein content in colonic samples was performed using the Quick Start™ Bradford Protein Assay (BIO-RAD, Hercules, CA, USA); the protein-dye complex absorbance was read using a spectrophotometer at 595 nm. GST activity was expressed as μmol/mg of protein per min.

Naphthol AS-D chloroacetate esterase assay

In order to identify neutrophil infiltration within the tissue, we used a commercially available kit (naphthol AS-D chloroacetate esterase; 91C, Sigma-Aldrich, Milan, Italy). This enzyme is considered specific for cells of granulocytic lineage and its sites of activity show bright red granulation. Seven-micron-thick sections of colon were used for this assay. Briefly, tissue sections were fixed in citrate-acetone-formaldehyde solution and assayed according to the manufacturer's protocols. Specimens were mounted with mounting media (glycerol-PBS, 9:1), examined by light microscope (ECLIPSE 90i, Nikon Instruments, Calenzano, Italy) and representative photomicrographs were taken by DS-5M digital camera (Nikon Instruments, Calenzano, Italy).

Table 2 Total macroscopic score parameters

Stool consistency	Bleeding	Colon damage score	Colon weight score	Colon length score	Maximum score
0 = formed	0 = absent	0 = no inflammation	0 = < 5% weight loss	0 = < 5% shortening	14
1 = loose	1 = present	1 = hyperemia	1 = 5%-14% weight loss	1 = 5%-14% shortening	
2 = liquid		2 = slight erosion	2 = 15%-24% weight loss	2 = 15%-24% shortening	
		3 = extensive erosion/ulceration	3 = 25%-35% weight loss	3 = 25%-35% shortening	
			4 = > 35% weight loss	4 = > 35% shortening	

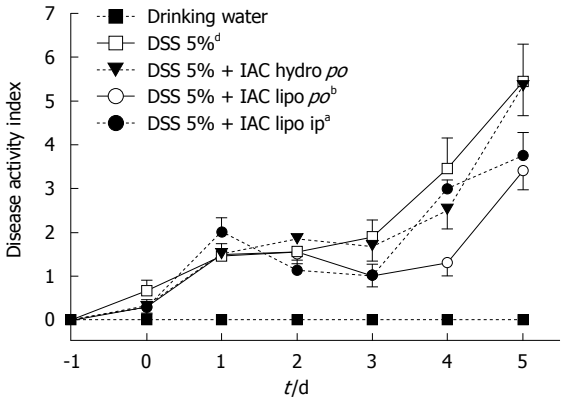

Figure 2 Effect of bis(1-hydroxy-2,2,6,6-tetramethyl-4-piperidinyl)decandioate 30 mg/kg on disease activity index in the different experimental groups. Data are expressed as mean ± SE; n = 5-12 rats per group. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* dextran sodium sulphate (DSS); $^{d}P < 0.001$ *vs* drinking water. Bis(1-hydroxy-2,2,6,6-tetramethyl-4-piperidinyl)decandioate (IAC) hydro: Hydrophilic form of IAC; IAC lipo: Lipophilic form of IAC.

Statistical analysis

Results are expressed as mean ± SE. Statistical analysis was performed using analysis of variance (one-way or two-way, as appropriate, with the Bonferroni's correction for multiple comparisons). A P value < 0.05 was considered significant. N refers to the number of animals used for each experiment (n = 8-16). Calculations were performed using GraphPad Prism™ (version 5.01, GraphPad Software Inc., San Diego, CA, USA).

RESULTS

Assessment of colitis

All rats with DSS colitis progressively lost weight and manifested bloody diarrhea. DAI was significantly increased together with all inflammatory parameters (Figures 2-6). On day 5 after DSS administration, the colonic mucosa of inflamed rats was edematous and erythematous with occasional areas of mucosal erosion. Compared to non-inflamed rats, the colon weight was decreased, the colon length was significantly shorter and a marked increase in total macroscopic damage score was noted (Figure 3, panel A). Colitis was characterized by mucosal ulceration, crypt dropout and a marked neutrophil infiltration extending throughout the mucosa and submucosal layers (Figure 5B) and by a 20-fold increase in microscopic damage score over the non-inflamed control animals (Figure 3, panel B); crypt abscesses and depletion of goblet cells were observed in some regions of colonic mucosa. A 3-fold increase in MPO activity was found (Figure 3, panel C) compared with healthy rats. Moreover, in DSS-treated animals, we detected neutrophil infiltration (red cells, Figure 6B) with respect to controls (Figure 6A), extending throughout the mucosa and submucosa. GST activity slightly decreased in inflamed animals as compared with healthy controls, although statistical significance was not achieved (1.40 ± 0.14 *vs* 1.27 ± 0.11).

Effect of IAC on DSS-induced colitis

Six-day treatment with lipophilic IAC (30 mg/kg, orally and ip) reduced intestinal inflammation and damage: indeed, treated rats had neither bloody diarrhea nor perianal injury and an improvement was observed in gross findings (clinical signs and symptoms of colitis) such as weight loss (Figure 2). Total macroscopic score was significantly improved by drug treatment (Figure 3, panel A). DSS-induced colon shrinkage and weight loss were significantly improved by IAC treatment (lipophilic) as compared to inflamed rats, and these features were similar to healthy controls (Figure 4). Moreover, lipophilic IAC (orally and ip) significantly decreased DSS-induced microscopic damage (Figure 3, panel B and Figure 5D), down-regulated MPO activity (Figure 3, panel C) and also minimized DSS-induced neutrophil infiltration within the colonic wall (Figure 6D).

In contrast, hydrophilic IAC 30 mg/kg orally was able to decrease only microscopic damage score, but substantially failed to protect the colon from DSS-induced damage (Figures 2-4, 5C and 6C).

GST activity was not significantly affected either by hydrophilic IAC *po* (1.27 ± 0.10 *vs* 1.27 ± 0.11 in DSS 5%) or lipophilic IAC *po* (1.28 ± 0.07 *vs* 1.27 ± 0.11 in DSS 5%) or ip (1.00 ± 0.07 *vs* 1.27 ± 0.11 in DSS 5%, P = 0.07).

DISCUSSION

This study shows that the lipophilic form of IAC at the dose of 30 mg/kg significantly ameliorates damage and inflammation in DSS-induced colitis, an experimental model of UC, and is far better than its hydrophilic form, since it positively affected all the parameters under scrutiny. This different activity on inflammation can be ascribed to the differing ability of the two distinct forms of IAC (hydrophilic *vs* lipophilic) to distribute in cell membranes and intra-/extra-cellular compartments; hydrophilic IAC may have a particular profile of absorption and distribution, leading to a lower concentration within areas of major damage.

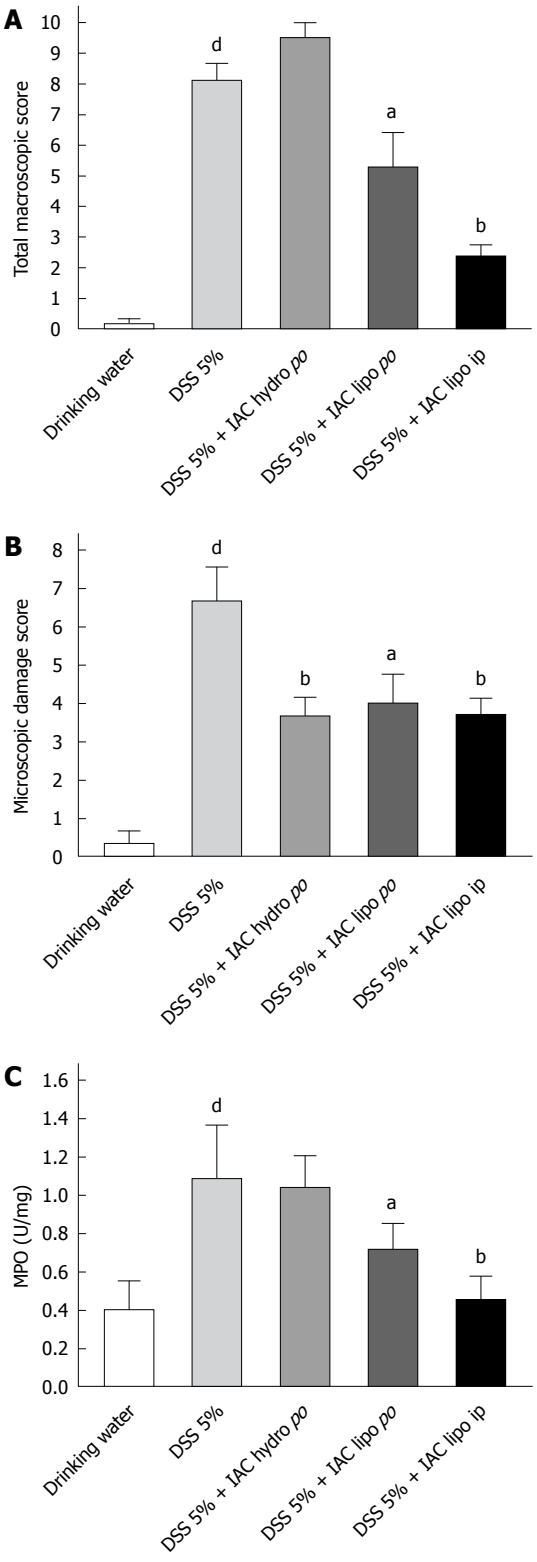

Figure 3 Effect of bis(1-hydroxy-2,2,6,6-tetramethyl-4-piperidinyl)decandioate 30 mg/kg (hydrophilic or lipophilic form) on total macroscopic (A) and microscopic damage score (B) and on myeloperoxidase activity (C) in the different experimental groups. Treatment with hydrophilic bis(1-hydroxy-2,2,6,6-tetramethyl-4-piperidinyl)decandioate (IAC) *po* decreased colonic microscopic damage but had no effect on macroscopic and myeloperoxidase (MPO) activity. Six-day treatment with lipophilic IAC *po* and ip significantly reduced intestinal damage induced by inflammation. Data are expressed as mean ± SE; n = 5-12 rats per group. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* dextran sodium sulphate (DSS); $^{d}P < 0.001$ *vs* drinking water. ICC hydro: Hydrophilic form of IAC; IAC lipo: Lipophilic form of IAC.

Different experimental models can be used for pathophysiological studies and, because of their similarity to human IBD (UC and CD), they represent useful tools to test the therapeutic potential of new drugs. These models allow the study of early events and interactions among different components of IBD and the identification of immunologic processes and genes determining susceptibility to inflammatory disorders. Although there is no unique model of IBD and the various experimental models differ in their pathophysiology, each of them can be useful to gain insight into the multi-factorial nature of IBD[10].

In DSS-induced colitis, as in UC, only the mucosa is affected by inflammation; early damage includes shortening and dropout of crypts in the left colon, particularly over lymphoid aggregates, progressing to focal ulceration, mononuclear cell and neutrophil infiltration.

Reactive oxygen, nitrogen and carbon species are produced and released during the acute phase of inflammation, resulting in epithelial damage with consequent activation of innate immune responses by luminal bacterial components and eventual activation of Th1 and later Th1/Th2 responses during chronic colitis[2,11].

In our study, 5-d treatment with 5% DSS induced weight loss and bloody diarrhea and caused a substantial degree of inflammation and tissue injury in the rat colon, which was edematous, erythematous and characterized by mucosal ulceration. Moreover, inflammation was associated with polymorphonuclear colonic infiltrate (histology, MPO activity and naphthol AS-D chloroacetate esterase assay). Indeed, within the bowel wall of IBD patients and of animals with experimental colitis, a massive infiltration of polymorphonuclear and mononuclear leukocytes, which may produce large amounts of free radicals, is commonly observed[1,9,21,22].

During DSS-induced colitis, we also studied GST activity. GST is a detoxification enzyme catalyzing the conjugation of reactive electrophiles with the thiol glutathione, providing cellular protection from highly reactive electrophiles[23]. A significant decrease in the GST activity was reported in patients with family history of colon cancer and polyps[24]; moreover, GST level changes have been observed both in IBD patients[25] and in some experimental models of colitis[23,26]. The only marginal (not statistically significant) decrease in GST activity observed by us on day 5 after DSS administration is actually in line with data obtained by Clapper *et al*[23], who showed cyclical changes in GST activity during acute DSS colitis, with a significant decrease in enzyme activity on days 2 and 7, while on day 5 they reported only marginally decreased GST activity, exactly as we did.

Treatment with lipophilic IAC (30 mg/kg, orally and ip) significantly ameliorated colonic damage and inflammation induced by DSS, decreased MPO activity and also minimized DSS-induced neutrophil infiltration within the colonic wall. These observations extend and corroborate our previous report that IAC is effective in DNBS-induced colitis[9], a quite different model where DNBS causes transmural immunological activation and inflam-

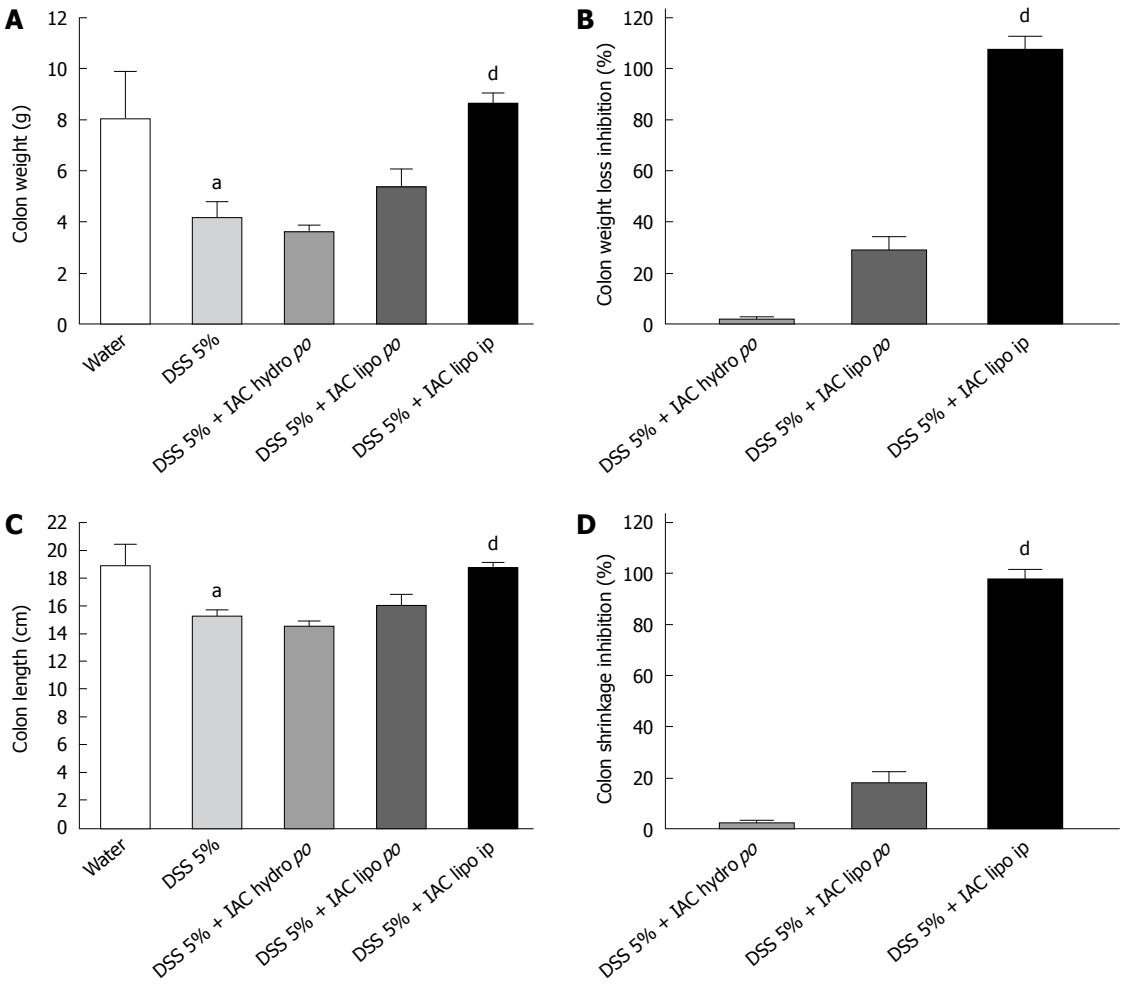

Figure 4 Effect of bis(1-hydroxy-2,2,6,6-tetramethyl-4-piperidinyl)decandioate 30 mg/kg (hydrophilic or lipophilic form) on dextran sodium sulphate-induced colon weight loss (absolute value, A, and % inhibition, B) and shrinkage (absolute values, C, and % inhibition, D) in the different experimental groups. Six-day treatment with lipophilic bis(1-hydroxy-2,2,6,6-tetramethyl-4-piperidinyl)decandioate (IAC) ip significantly inhibited colon weight loss and colon shrinkage induced by inflammation, while the hydrophilic form had no effect. Data are expressed as mean ± SE; n = 5-12 rats per group. $^{a}P < 0.05$ *vs* water; $^{d}P < 0.01$ *vs* dextran sodium sulphate (DSS). IAC hydro: Hydrophilic form of IAC; IAC lipo: Lipophilic form of IAC.

mation resembling CD. In our hands, lipophilic IAC was slightly more effective ip than orally, probably because it undergoes protonation at low gastric pH after oral administration and is more quickly excreted from the gut (the protonated form has higher polarity). For clinical use, it may be necessary to protect IAC from low gastric pH using a specific formulation.

Thus, lipophilic IAC is protective both in DNBS and DSS experimental models of inflammation and has a wide spectrum of activity; its lipophilic form (ip) significantly reduced DAI (30%), macroscopic and microscopic damage (70% and 45%, respectively) and decreased MPO activity (58%) in DSS-induced colitis. Likewise, in DNBS-induced colitis, treatment with lipophilic IAC can decrease macroscopic and microscopic damage (55% and 46%, respectively), reduce MPO and tumor necrosis factor-α tissue levels (80% and 30%, respectively) and lipidic peroxidation (40%).

Notably, in both the DSS and DNBS models[9], IAC is able to significantly decrease MPO activity and neutrophil infiltration within the bowel wall. In both experimental models, as in several human inflammatory diseases such as rheumatoid arthritis and IBD, infiltrating neutrophils are the major candidate for the production of reactive oxygen radicals[27]. Oxidative damage may represent a pathogenic factor in IBD because intestinal inflammation is accompanied by increased production of reactive oxygen and nitrogen species and an imbalanced antioxidant response[1,28,29]. Indeed, free radical production is a key mechanism for the appearance and the maintenance of colonic inflammation in experimental models of colitis[7,30,31].

Thus, we can hypothesize that IAC exerts its protective effects by reducing inflammatory neutrophil infiltrate and by scavenging reactive oxygen species produced by infiltrating cells which during inflammation contribute to the recruitment and activation of further neutrophils, and by counteracting this self-sustaining cycle of oxidant production, which propagates inflammation and tissue damage. This hypothesis of reduced oxidative damage by IAC is not contrary to its lack of activity on GST levels because, in our hands, GST levels were only marginally affected by DSS-induced inflammation. It has been shown that treatment with antioxidant compounds and radical scavengers exerts a protective effect in several models of

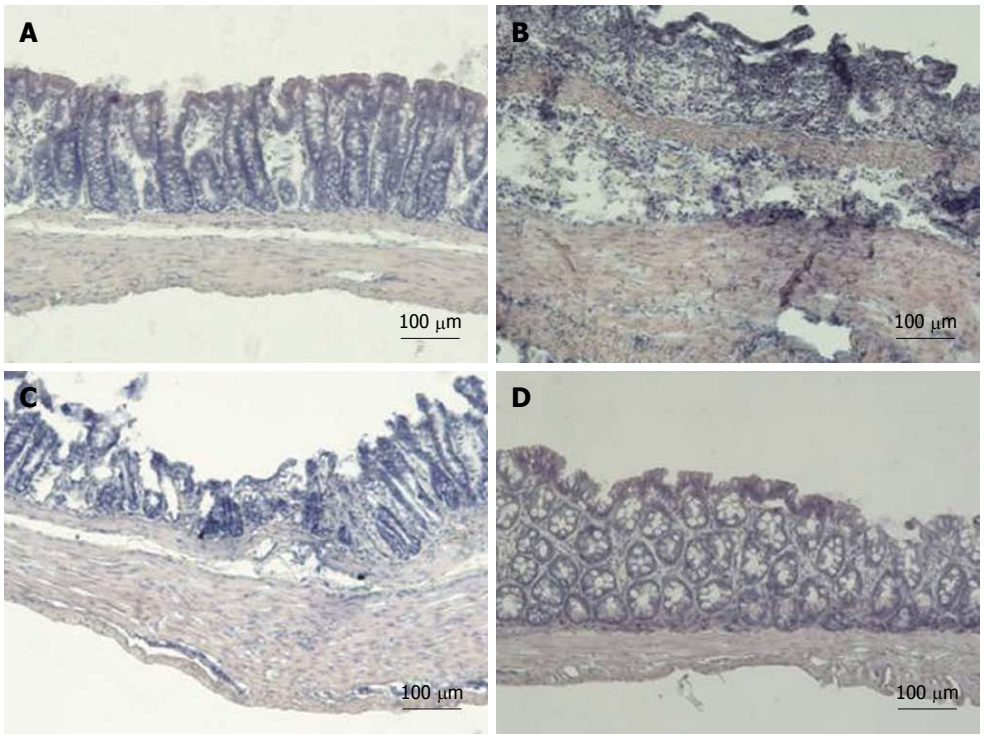

Figure 5 Representative examples of cross sections of distal colon. A: From a non-inflamed rat [drinking water + bis(1-hydroxy-2,2,6,6-tetramethyl-4-piperidinyl) decandioate (IAC) vehicle orally]; B: From an inflamed rat [dextran sodium sulphate (DSS) 5% in drinking water + IAC vehicle orally]. Note the dramatic loss of mucosal architecture with crypt dropout and the granulocyte infiltrate extending throughout the mucosa and submucosa; C, D: Cross sections of distal colon from an inflamed rat treated with hydrophilic (C) and lipophilic (D) IAC 30 mg/kg orally (C) and intraperitoneally (D). Lipophilic (*po*, not shown here, and ip) IAC 30 mg/kg decreased the microscopic damage produced by DSS, facilitating mucosal healing, reducing inflammatory cells infiltration and muscle thickening (panel D). Hydrophilic IAC *po* failed to protect the colon from the damage induced by DSS (panel C).

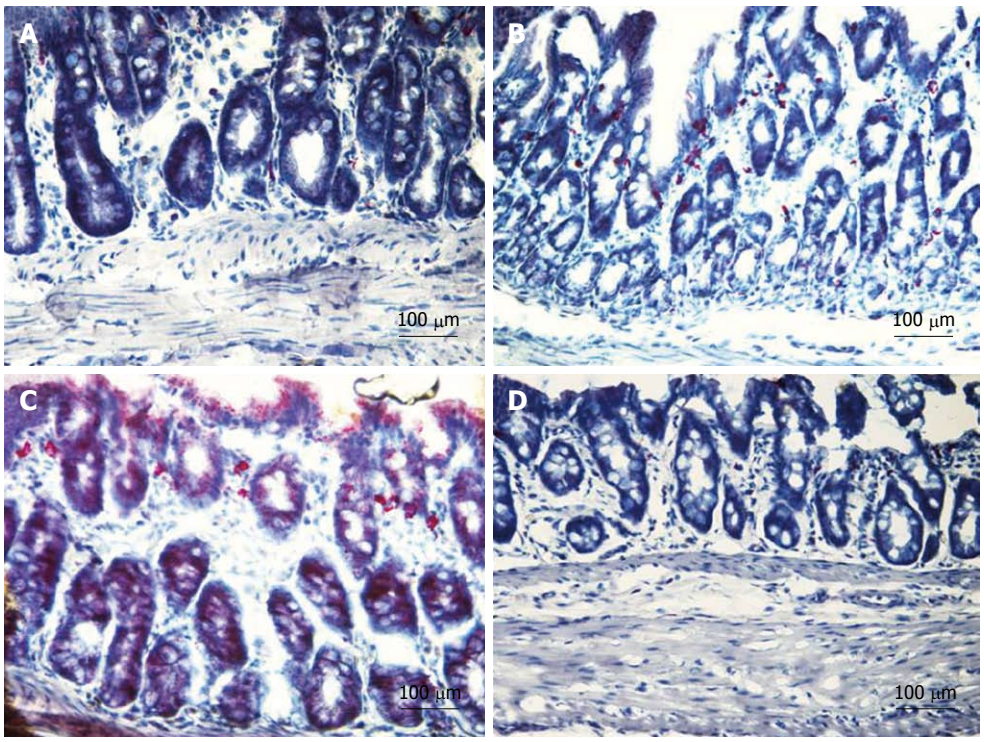

Figure 6 Naphthol AS-D chloroacetate esterase positivity (red) in cross section of distal colon. A: Tissue sections obtained from non-inflamed rats showed occasional red staining indicating a low presence of neutrophils within the bowel wall under physiological conditions; B: From a rat with colitis (5% dextran sodium sulphate in drinking water, panel B). Compared to non-inflamed rats, tissue from rats with colitis showed a massive neutrophil infiltration extending throughout the mucosa (note the scattered degranulation within the crypts and submucosa); C, D: Treatment with bis(1-hydroxy-2,2,6,6-tetramethyl-4-piperidinyl)decandioate (IAC) 30 mg/kg hydrophilic form *po* (C) was unable to suppress neutrophil infiltration especially in the mucosa. Treatment with lipophilic IAC both *po* (not shown) and ip (D) almost completely suppressed neutrophil infiltration within the colonic wall.

intestinal inflammation[9,32,33]: Cuzzocrea *et al*[7,34] demonstrated that treatment with antioxidants such as tempol and M40403, a superoxide dismutase mimetic, ameliorated TNBS/DNBS experimental colitis, probably by limiting leukocyte recruitment. However, cyclic nitroxides such as tempol are very persistent in water or organic solutions, but when used *in vivo* or in a biological sample are reduced to the parent hydroxylamine by several enzymatic processes mainly involving ascorbate or glutathione. IAC is more stable in physiological solutions and possesses a stronger antioxidant capability than that of the aforementioned cyclic nitroxides; it is easily distributed through cell membranes and intra-/extra-cellular compartments, thus it can directly react with oxidant molecules within the cell, where free radicals are produced[35]. In DNBS-induced colitis, IAC seems to display higher activity than tempol, at least in reducing MPO activity and neutrophil infiltration[7]. Recently, the lipophilic form of IAC was used in different experimental disease models all characterized by oxidative stress (e.g. nonobese mouse diabetes model[15] and a rat model of transient middle cerebral artery occlusion[36]), as well as *in vitro*[37], with positive results.

Notably, IAC is a low molecular weight radical scavenger which can rapidly react with most carbon-, nitrogen- and oxygen-centered radicals of biological interest, including peroxyl, superoxide, and peroxynitrite radicals[13]. Its antioxidant activity is a direct effect of the molecule itself; its activity is due to hydroxylic hydrogen transfer to peroxyl radicals, which generates the corresponding nitroxide, unable to propagate the autoxidation chain. Its peculiar physico-chemical properties affect its partition properties across cell membranes and both intra- and extra-cellular compartments: the free form is highly lipophilic (the calculated logP is 4.01) and easily crosses the cell membrane, allowing distribution to any compartment where the production of free radicals occurs, but it is also in equilibrium with the protonated form, which administered to a biological system is completely water-soluble and distributes in the extra-cellular compartments[13].

In conclusion, our data show that treatment with the lipophilic (but not the hydrophilic) form of the radical scavenger IAC, at the dose of 30 mg/kg, ameliorates DSS-induced colitis in rats. These results, taken together with our previous data showing a protective effect of lipophilic IAC in DNBS-induced colitis, provide further evidence of the involvement of reactive oxygen species in inflammation and support the concept that antioxidant therapy may have an important role in treatment of IBD.

ACKNOWLEDGMENTS

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COMMENTS

Background

Ulcerative colitis (UC) and Crohn's disease are chronic inflammatory bowel diseases (IBDs) of unclear etiology: environmental and genetic factors regulating mucosal immune response, mucosal barrier function and response to intestinal microflora are all thought to contribute to the pathogenesis of these diseases, characterized by mucosal inflammatory infiltrates, intestinal barrier function alteration and erosive loss of mucosa and submucosa. Indeed, the intestinal mucosa of patients with IBD is characterized by radical species overproduction and imbalance of the most important antioxidants leading to oxidative damage.

Research frontiers

Over the last two decades, the incidence and the prevalence of IBD seem to have increased; IBDs cause a large number of hospitalizations for the patients affected. Many drugs are used to treat IBD, given for a variety of reasons: to suppress inflammation in patients with active disease, to prevent flare-ups in those with inactive disease, to control symptoms such as pain or diarrhea or to replace or supplement essential nutrients which are poorly absorbed because of extensive disease or surgery. However, the etiology of IBD is still unknown and new therapeutic options are needed, as available drugs are still unsatisfactory to treat and heal IBD.

Innovations and breakthroughs

It is generally hypothesized that oxidative stress is a potential etiological and/or triggering factor for IBD, because the detrimental effects of reactive oxygen molecules have been well established in the inflammation process. Antioxidant compounds and free radical scavengers have been shown to improve colitis in several experimental models suggesting an important role of reactive oxygen species in intestinal inflammation. This study clearly shows the effect of an antioxidant molecule in an experimental model of colitis which resembles human UC, indicating a potential clinical application for this class of compounds in treating IBD.

Applications

The protective effect shown by antioxidant therapy in this experimental model of colitis indicates a potential clinical application for this class of compounds in treating IBD. However, several clinical studies have been largely disappointing, probably due to the inability of the antioxidant to reach sufficient concentrations at the inflammation site. Lipophilic bis(1-hydroxy-2,2,6,6-tetramethyl-4-piperidinyl)decandioate (IAC) is easily distributed through cell membranes and intra-/extra-cellular compartments and directly reacts with oxidant molecules within the cell, where free radicals are produced. For clinical use, it may be necessary to protect IAC from low gastric pH using a specific formulation in order to improve its bioavailability.

Peer review

The study is well done with adequate supporting documentation, controls and references. The difference in activity between the lipophilic and hydrophilic forms of IAC in suppressing dextran sodium sulphate-induced colitis is a novel and important finding.

REFERENCES

1 **Kruidenier L**, Kuiper I, Lamers CB, Verspaget HW. Intestinal oxidative damage in inflammatory bowel disease: semi-quantification, localization, and association with mucosal antioxidants. *J Pathol* 2003; **201**: 28-36
2 **Strober W**, Fuss IJ, Blumberg RS. The immunology of mucosal models of inflammation. *Annu Rev Immunol* 2002; **20**: 495-549
3 **Naito Y**, Takagi T, Yoshikawa T. Molecular fingerprints of neutrophil-dependent oxidative stress in inflammatory bowel disease. *J Gastroenterol* 2007; **42**: 787-798
4 **Rezaie A**, Parker RD, Abdollahi M. Oxidative stress and pathogenesis of inflammatory bowel disease: an epiphenomenon or the cause? *Dig Dis Sci* 2007; **52**: 2015-2021
5 **Babbs CF**. Oxygen radicals in ulcerative colitis. *Free Radic Biol Med* 1992; **13**: 169-181
6 **Simmonds NJ**, Rampton DS. Inflammatory bowel disease--a radical view. *Gut* 1993; **34**: 865-868
7 **Cuzzocrea S**, McDonald MC, Mazzon E, Dugo L, Lepore V, Fonti MT, Ciccolo A, Terranova ML, Caputi AP, Thiemermann C. Tempol, a membrane-permeable radical scavenger, reduces dinitrobenzene sulfonic acid-induced colitis. *Eur J Pharmacol* 2000; **406**: 127-137
8 **Ishihara T**, Tanaka K, Tasaka Y, Namba T, Suzuki J, Ishihara T, Okamoto S, Hibi T, Takenaga M, Igarashi R, Sato K, Mizushima Y, Mizushima T. Therapeutic effect of lecithinized

superoxide dismutase against colitis. *J Pharmacol Exp Ther* 2009; **328**: 152-164
9 **Vasina V**, Broccoli M, Ursino MG, Bellot SF, Soleti A, Paolini M, De Ponti F. Effects of the non-peptidyl low molecular weight radical scavenger IAC in DNBS-induced colitis in rats. *Eur J Pharmacol* 2009; **614**: 137-145
10 **Elson CO**, Sartor RB, Tennyson GS, Riddell RH. Experimental models of inflammatory bowel disease. *Gastroenterology* 1995; **109**: 1344-1367
11 **Dieleman LA**, Palmen MJ, Akol H, Bloemena E, Peña AS, Meuwissen SG, Van Rees EP. Chronic experimental colitis induced by dextran sulphate sodium (DSS) is characterized by Th1 and Th2 cytokines. *Clin Exp Immunol* 1998; **114**: 385-391
12 **Kim HS**, Berstad A. Experimental colitis in animal models. *Scand J Gastroenterol* 1992; **27**: 529-537
13 **Valgimigli L**, Pedulli GF, Paolini M. Measurement of oxidative stress by EPR radical-probe technique. *Free Radic Biol Med* 2001; **31**: 708-716
14 **Verma-Gandhu M**, Verdu EF, Bercik P, Blennerhassett PA, Al-Mutawaly N, Ghia JE, Collins SM. Visceral pain perception is determined by the duration of colitis and associated neuropeptide expression in the mouse. *Gut* 2007; **56**: 358-364
15 **Novelli M**, D'Aleo V, Lupi R, Paolini M, Soleti A, Marchetti P, Masiello P. Reduction of oxidative stress by a new low-molecular-weight antioxidant improves metabolic alterations in a nonobese mouse diabetes model. *Pancreas* 2007; **35**: e10-e17
16 **Cooper HS**, Murthy SN, Shah RS, Sedergran DJ. Clinicopathologic study of dextran sulfate sodium experimental murine colitis. *Lab Invest* 1993; **69**: 238-249
17 **Egger B**, Bajaj-Elliott M, MacDonald TT, Inglin R, Eysselein VE, Büchler MW. Characterisation of acute murine dextran sodium sulphate colitis: cytokine profile and dose dependency. *Digestion* 2000; **62**: 240-248
18 **Diaz-Granados N**, Howe K, Lu J, McKay DM. Dextran sulfate sodium-induced colonic histopathology, but not altered epithelial ion transport, is reduced by inhibition of phosphodiesterase activity. *Am J Pathol* 2000; **156**: 2169-2177
19 **Boughton-Smith NK**, Wallace JL, Whittle BJ. Relationship between arachidonic acid metabolism, myeloperoxidase activity and leukocyte infiltration in a rat model of inflammatory bowel disease. *Agents Actions* 1988; **25**: 115-123
20 **Habig WH**, Pabst MJ, Jakoby WB. Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. *J Biol Chem* 1974; **249**: 7130-7139
21 **Kitahora T**, Suzuki K, Asakura H, Yoshida T, Suematsu M, Watanabe M, Aiso S, Tsuchiya M. Active oxygen species generated by monocytes and polymorphonuclear cells in Crohn's disease. *Dig Dis Sci* 1988; **33**: 951-955
22 **Damiani CR**, Benetton CA, Stoffel C, Bardini KC, Cardoso VH, Di Giunta G, Pinho RA, Dal-Pizzol F, Streck EL. Oxidative stress and metabolism in animal model of colitis induced by dextran sulfate sodium. *J Gastroenterol Hepatol* 2007; **22**: 1846-1851
23 **Clapper ML**, Adrian RH, Pfeiffer GR, Kido K, Everley L, Cooper HS, Murthy S. Depletion of colonic detoxication enzyme activity in mice with dextran sulphate sodium-induced colitis. *Aliment Pharmacol Ther* 1999; **13**: 389-396
24 **Szarka CE**, Pfeiffer GR, Hum ST, Everley LC, Balshem AM, Moore DF, Litwin S, Goosenberg EB, Frucht H, Engstrom PF. Glutathione S-transferase activity and glutathione S-transferase mu expression in subjects with risk for colorectal cancer. *Cancer Res* 1995; **55**: 2789-2793
25 **Duncan H**, Swan C, Green J, Jones P, Brannigan K, Alldersea J, Fryer AA, Strange RC. Susceptibility to ulcerative colitis and Crohn's disease: interactions between glutathione S-transferase GSTM1 and GSTT1 genotypes. *Clin Chim Acta* 1995; **240**: 53-61
26 **Nieto N**, Torres MI, Fernández MI, Girón MD, Ríos A, Suárez MD, Gil A. Experimental ulcerative colitis impairs antioxidant defense system in rat intestine. *Dig Dis Sci* 2000; **45**: 1820-1827
27 **Grisham MB**, Granger DN. Neutrophil-mediated mucosal injury. Role of reactive oxygen metabolites. *Dig Dis Sci* 1988; **33**: 6S-15S
28 **Grisham MB**. Oxidants and free radicals in inflammatory bowel disease. *Lancet* 1994; **344**: 859-861
29 **Kruidenier L**, Kuiper I, Van Duijn W, Mieremet-Ooms MA, van Hogezand RA, Lamers CB, Verspaget HW. Imbalanced secondary mucosal antioxidant response in inflammatory bowel disease. *J Pathol* 2003; **201**: 17-27
30 **Keshavarzian A**, Morgan G, Sedghi S, Gordon JH, Doria M. Role of reactive oxygen metabolites in experimental colitis. *Gut* 1990; **31**: 786-790
31 **Seguí J**, Gil F, Gironella M, Alvarez M, Gimeno M, Coronel P, Closa D, Piqué JM, Panés J. Down-regulation of endothelial adhesion molecules and leukocyte adhesion by treatment with superoxide dismutase is beneficial in chronic immune experimental colitis. *Inflamm Bowel Dis* 2005; **11**: 872-882
32 **Oz HS**, Chen TS, McClain CJ, de Villiers WJ. Antioxidants as novel therapy in a murine model of colitis. *J Nutr Biochem* 2005; **16**: 297-304
33 **Kruidenier L**, van Meeteren ME, Kuiper I, Jaarsma D, Lamers CB, Zijlstra FJ, Verspaget HW. Attenuated mild colonic inflammation and improved survival from severe DSS-colitis of transgenic Cu/Zn-SOD mice. *Free Radic Biol Med* 2003; **34**: 753-765
34 **Cuzzocrea S**, Mazzon E, Dugo L, Caputi AP, Riley DP, Salvemini D. Protective effects of M40403, a superoxide dismutase mimetic, in a rodent model of colitis. *Eur J Pharmacol* 2001; **432**: 79-89
35 **Valgimigli L**, Valgimigli M, Gaiani S, Pedulli GF, Bolondi L. Measurement of oxidative stress in human liver by EPR spin-probe technique. *Free Radic Res* 2000; **33**: 167-178
36 **Nurmi A**, Miettinen TK, Puoliväli J, Pussinen R, Soleti A, Bagate K, Riccardino F, Grundy RI, Yrjänheikki J, Canistro D, Paolini M. Neuroprotective properties of the non-peptidyl radical scavenger IAC in rats following transient focal cerebral ischemia. *Brain Res* 2008; **1207**: 174-181
37 **D'Aleo V**, Del Guerra S, Martano M, Bonamassa B, Canistro D, Soleti A, Valgimigli L, Paolini M, Filipponi F, Boggi U, Del Prato S, Lupi R. The non-peptidyl low molecular weight radical scavenger IAC protects human pancreatic islets from lipotoxicity. *Mol Cell Endocrinol* 2009; **309**: 63-66

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ORIGINAL ARTICLE

Effects of protein deprivation and re-feeding on P2X2 receptors in enteric neurons

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Abstract

AIM: To investigate the effects of malnutrition and re-feeding on the P2X2 receptor, nitric oxide synthase (NOS), calretinin, calbindin and choline acetyltransferase (ChAT) in neurons of the rat ileum.

METHODS: We analyzed the co-localization, numbers and sizes of P2X2-expressing neurons in relation to NOS-immunoreactive (IR), calbindin-IR, ChAT-IR, and calretinin-IR neurons of the myenteric and submucosal plexus. The experimental groups consisted of: (1) rats maintained on normal feed throughout pregnancy until 42 d post-parturition (N); (2) rats deprived of protein throughout pregnancy and 42 d post-parturition (D); and (3) rats undernourished for 21 d post-parturition and then given a protein diet from days 22 to 42 (DR). The myenteric and submucosal plexuses were evaluated by double labeling by immunohistochemical methods for P2X2 receptor, NOS, ChAT, calbindin and calretinin.

RESULTS: We found similar P2X2 receptor immunoreactivity in the cytoplasm and surface membranes of myenteric and submucosal neurons from the N, D and DR groups. Double labeling of the myenteric plexus demonstrated that approximately 100% of NOS-IR, calbindin-IR, calretinin-IR and ChAT-IR neurons in all groups also expressed the P2X2 receptor. In the submucosal plexus, the calretinin-IR, ChAT-IR and calbindin-IR neurons were nearly all immunoreactive for the P2X2 receptor. In the myenteric plexus, there was a 19% increase in numbers per cm^2 for P2X2 receptor-IR neurons, 64% for NOS-IR, 84% for calretinin-IR and 26% for ChAT-IR neurons in the D group. The spatial density of calbindin-IR neurons, however, did not differ among the three groups. The submucosal neuronal density increased for calbindin-IR, calretinin-IR and ChAT-IR neurons. The average size of neurons in the myenteric plexus neurons in the D group was less than that in the controls and, in the re-fed rats; there was a 34% reduction in size only for the calretinin-IR neurons.

CONCLUSION: This work demonstrates that expression of the P2X2 receptor is present in inhibitory, intrinsic primary afferent, cholinergic secretomotor and vasomotor neurons. Undernutrition affected P2X2 receptor expression in the submucosal plexus, and neuronal and size. These changes were rescued in the re-fed rats.

Key words: Chemical coding; Myenteric neurons; Submucosal neurons; Undernutrition

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INTRODUCTION

ATP is known to be a co-transmitter in the nervous system and a ligand of the P2X receptor family, which is made up of seven known receptor subunits (PX_{1-7})[1]. In the myenteric plexus, electrophysiological studies have found P2X receptors in 80%-90% of neurons[2]. P2X receptors play an important role in synaptic transmission within the neural pathways and mediate intestinal motility[3-5]. Immunohistochemical studies have documented the distribution of P2X receptors in the enteric nervous system of guinea pigs[6-11], rats[12-14] and mice[15,16]. There is only one earlier study in which the authors have reported expression of $P2X_2$ receptor in calretinin and calbindin neurons in the ileal myenteric plexus of rats[12].

The effects of undernutrition on enteric and other autonomic neurons have been investigated[17-25]. In the enteric nervous system, a 27% decrease in the number of enteric neurons in the jejunum of rats submitted to severe prenatal malnutrition has been reported[18], and a mean neuronal loss of 13% in the myenteric plexus of the proximal colon has been observed after malnutrition[20]. Experiments that have examined the effect of re-feeding on enteric neuronal number suggest that, when neurons are reduced in number by undernutrition, they do not recover[18]. However, other reports have described a 15% decrease in the sizes of myenteric neurons from the large intestine of pre- and postnatally protein-deprived animals, as well as recovery of normal size after re-feeding[21]. A 45% size reduction in enteric neurons of the small intestine has also been observed following undernutrition[22].

The present work analyzed the effects of pre- and postnatal protein undernutrition and postnatal re-feeding on neurons immunoreactive for the $P2X_2$ receptor, by specifically examining the expression of nitric oxide synthase (NOS), calretinin, calbindin and choline acetyltransferase (ChAT) in these neurons, as well as neuronal density and somatic size in the myenteric and submucosal plexuses in the rat ileum.

MATERIALS AND METHODS

Experimental animals

The study was conducted according to current legislation on animal experiments of the Biomedical Science Institute of the University of São Paulo. Young male and female Wistar rats (200-240 g body weight) were mated. After conception, which was assumed to have occurred when vaginal sperm plugs were found, the females were placed in individual cages. During pregnancy, the nourished mothers received an AIN-93G normal protein diet (protein, 20%; fat, 7%; carbohydrate, 20% and fiber, 5%), and the undernourished mothers received the AIN-93G diet with low protein (protein, 5%; fat, 7%; carbohydrate, 20% and fiber, 5%) (Rhoster Indústria e Comércio Ltda, São Paulo, Brazil). The rats were maintained under standard conditions at 21℃, with a 12-h light/dark cycle, and all groups were supplied with water *ad libitum*. After parturition, the dams and pups received the same diet that the dam had during pregnancy. Only the male animals in the litters were used for experimentation. Females remained in the litters but were not investigated. There were three experimental groups. The first group of rats was maintained on normal feed throughout pregnancy until examined at 42 d (P42) (N, n = 5). The second group was protein-deprived throughout pregnancy and postnatally for 42 d (P42) (D, n = 5). The third group of rats was the deprived plus re-feeding group (DR, n = 5), in which animals were undernourished until P21, and then received the AIN-93G normal protein diet from P22 to P42[21,22]. At P42, animals were weighed and euthanized in a CO_2 chamber and the anterior abdominal wall was opened. The small intestine was removed and washed in PBS. The surface area of the small intestine was measured using a planimeter.

Immunohistochemistry

Fresh segments of ileum were removed from each animal of the N, D and DR groups and placed in PBS (0.15 mol/L NaCl in 0.01 mol/L sodium phosphate buffer, pH 7.2) that contained nicardipine (10^{-6} mol/L; Sigma, St Louis, MO, USA) to inhibit tissue contraction. The dissected pieces were opened along the mesenteric border and cleaned of their contents using PBS. They were then pinned out tautly, mucosa-side down, onto a balsa-wood board and fixed overnight at 4℃ in paraformaldehyde in 0.2 mol/L sodium phosphate buffer (pH 7.3). The next day, the tissue was cleared of fixative with three 10-min washes in 100% DMSO, followed by three 10-min washes in PBS. All tissue was stored at 4℃ in PBS that contained sodium azide (0.1%). The fixed tissue was dissected and the mucosa, submucosa and circular layers were removed to obtain longitudinal muscle-myenteric plexus whole mounts. In the second type of preparation, the mucosa and muscularis externa were removed to reveal the intact submucous layer. Whole-mount preparations of the myenteric and submucosa of the ileum were preincubated in 10% normal horse serum in PBS that contained 1.5% Triton X-100 for 30 min at room temperature, to reduce non-specific binding and to permeabilize the tissue (Table 1). To localize $P2X_2$ receptor immunoreactivity, we used a rabbit antiserum raised against amino acid sequence 457-472 of the rat $P2X_2$ receptor, with a single Cys extension at the N-terminal (AB5244; Chemicon, Temecula, CA, USA). Incubation was for 48 h at 4℃ at a dilution of 1:120 in 10% normal horse serum in PBS that contained 1.5% Triton X-100. Double labeling was achieved using combina-

Table 1 Characteristics of primary and secondary antibodies

Tissue antigen	Host	Dilution	Code and reference
NOS	Sheep	1:2000	H205
Calbindin	Mouse	1:500	Swant 300
Calretinin	Goat	1:100	CG1 Swant
ChAT	Goat	1:50	Chemicon
Donkey anti-rabbit IgG Alexa 488		1:500	Molecular probes
Donkey anti-sheep IgG Alexa 594		1:100	Molecular probes
Donkey anti-mouse IgG Alexa 594		1:200	Molecular probes

NOS: Nitric oxide synthase; ChAT: Choline acetyltransferase.

tions of antisera (Table 1). Following incubation in primary antisera, tissue was given three 10-min washes in PBS and incubated in a mixture of secondary antibodies (Table 1). Further 10-min washes in PBS were made before tissue was mounted in glycerol buffered with 0.5 mol/L sodium carbonate buffer (pH 8.6).

Imaging

Preparations were examined on a Leica microscope equipped with the appropriate filters for Alexa 488 (450-490 nm excitation filter and 515-565 nm emission filter) and Alexa 594 (530-585 nm excitation filter and 615 nm emission filter). Images were recorded using an Image-Pro-Plus-coupled camera and Image-Pro Plus software (Media Cybernetics, Bethesda, MD, USA). Preparations were also analyzed using confocal microscopy on a Zeiss confocal scanning laser system installed on a Zeiss Axioplan 2 microscope (Carl Zeiss). The system had a krypton/argon laser for differential visualization of the fluorophores using a 488-nm excitation filter and a 522/535-nm emission filter for 488 and 568 nm excitation filters and a 605/632 nm emission filter for Alexa 594. The images were 512 × 512 pixels in size and the thickness of each optical section was 0.5 μm. Immunoreactive cells were scanned as a series of optical sections with a center spacing of 0.2 μm. Confocal images were collected using LSM 5 Image Zeiss processing software (Carl Zeiss MicroImaging, Germany). Images were further processed using Corel Photo Paint and Corel Draw software programs (Corel Corporation).

Quantitative analyses

The proportions of neurons in which antigen immunoreactivity was co-localized were determined by examining double-labeled neurons. Neurons were first located by the presence of a fluorophore that labeled one antigen, and then the filter was switched to determine whether the neuron was labeled for a second antigen, located with a second fluorophore of a different color. In this way, proportions of neurons labeled for pairs of antigens were determined. The cohort size was 100 neurons and data were collected from preparations obtained from at least four animals. The percentage of neurons immunoreactive to a second neurochemical was calculated and expressed as mean ± SE. The numbers of P2X$_2$-immunoreactive (IR), NOS-IR, calbindin-IR, calretinin-IR and ChAT-IR neurons and nerve cell perikarya were measured by examining the whole-mount preparations under a binocular microscope at a magnification of 100 ×. All neurons present in each 1 cm^2 were counted. The nerve cell perikarya profiles area, major axes, and minor axes of 50 nerve cell perikarya from each animal were obtained on a semiautomatic morphometry device, the Image-Pro Plus Program.

Table 2 Body weight and small-intestinal area of pre- and postnatal protein-deprived rats at 42 d postnatal

	N	D	DR
Body weight (g)	160 ± 10	40 ± 18[a]	100 ± 22
Area of small intestine (cm^2)	20.41 ± 3.7	13.3 ± 0.6[a]	16.5 ± 0.4

[a]$P < 0.05$ *vs* protein deprived, Tukey's test for multiple comparisons.

Statistical analysis

mean ± SE were calculated and compared by analysis of variance and Tukey test for multiple comparisons, as appropriate. The level of significance was set at $P < 0.05$.

RESULTS

The mean body weight of animals of the N group (160 ± 10 g) was approximately 400% greater than that of the D group (40 ± 18 g). The body weight of the DR group (100 ± 22 g) was restored to within 20% of normal at P42 ($P < 0.05$). The small intestine area of the D group was 34% less ($P < 0.05$) than that of the N group, and there was no statistical difference between the N and DR groups (Table 2).

The qualitative results demonstrated that P2X$_2$ receptor immunoreactivity was found in the myenteric and submucosal plexuses of the ileum of all groups. Positive labeling was seen in the cytoplasm and surface membranes of most nerve cells of the nourished, undernourished and refed groups (Figure 1). The labeling intensity of the P2X$_2$ receptor in the myenteric and submucosal ganglia of the N, D and DR groups was similar. Double-labeling studies were conducted to identify neurons that had P2X$_2$ immunoreactivity co-localized with NOS, calbindin, calretinin and ChAT in ileal myenteric neurons (Figure 1), and calbindin, calretinin and ChAT in the ileal submucosal plexus of the N, D and DR groups (Figure 2). In all groups, the cellular morphology of the myenteric plexus showed that NOS-IR neurons had a Dogiel Type I morphology, while calretinin-IR neurons exhibited Dogiel Type II morphology, and calbindin-IR neurons had both small and large Dogiel Type II neurons. In the submucosal plexus, calbindin-IR neurons had Dogiel Type II morphology. The intensity of ChAT immunoreactivity was reduced in some neurons of the myenteric and submucosal ganglia of undernourished rats.

Co-localization

The quantitative results revealed that, in the myenteric plexuses, the majority of NOS-IR, calbindin-IR, calretinin-

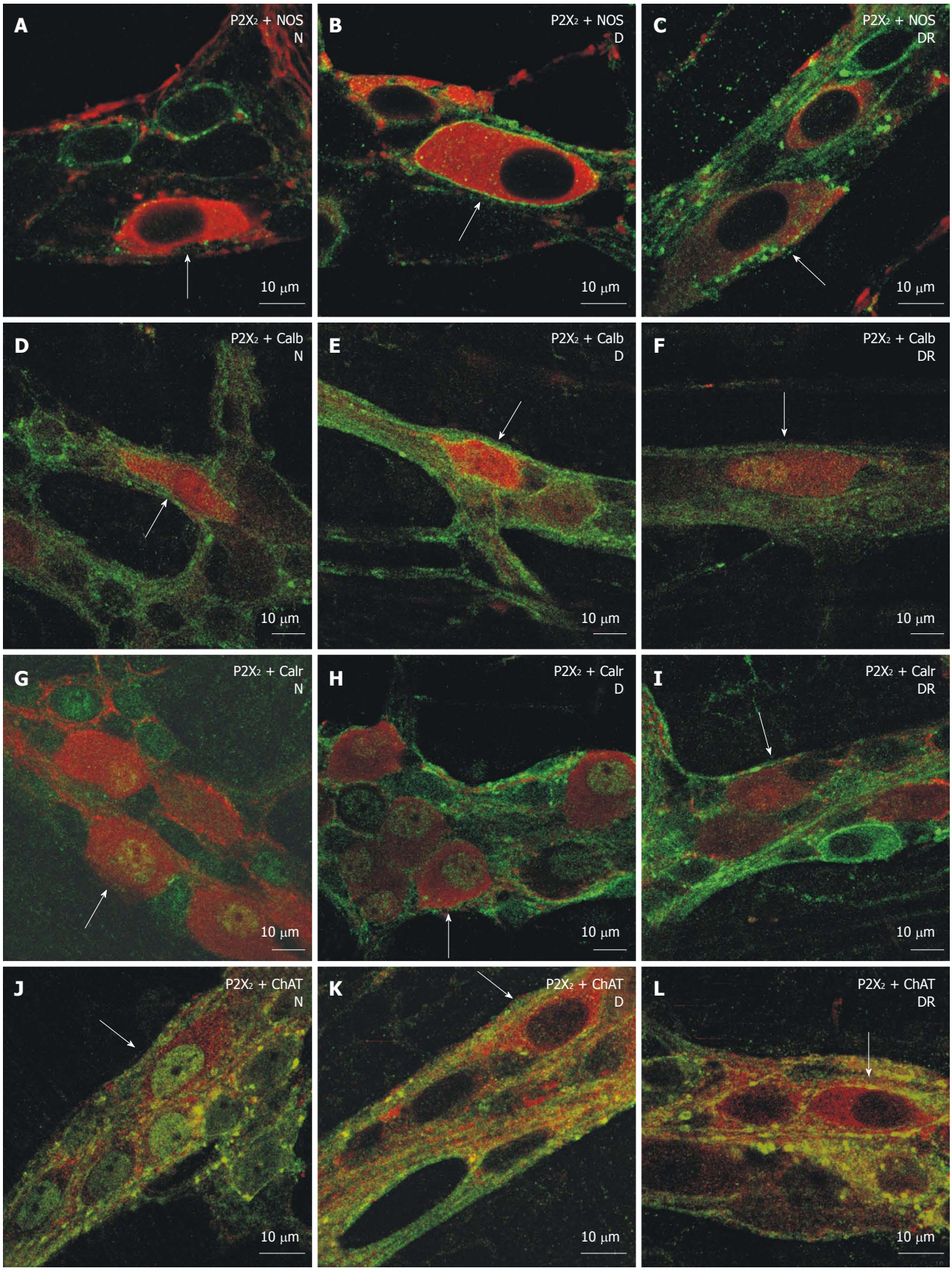

Figure 1 Co-localization of P2X2 receptor immunoreactivity with nitric oxide synthase, calbindin, calretinin and choline acetyltransferase immunoreactivity in the ileal myenteric plexus in the N, D and DR groups. A-C: P2X2 receptor-IR (green) co-localized with nitric oxide synthase (NOS)-IR (red); D-F: P2X2 receptor-IR (green) co-localized with calbindin-IR (red); G-I: P2X2 receptors (green) co-localized with calretinin (red); J-L: P2X2 receptors (green) co-localized with choline acetyltransferase (ChAT) (red). Double-labeled neurons are indicated by arrows.

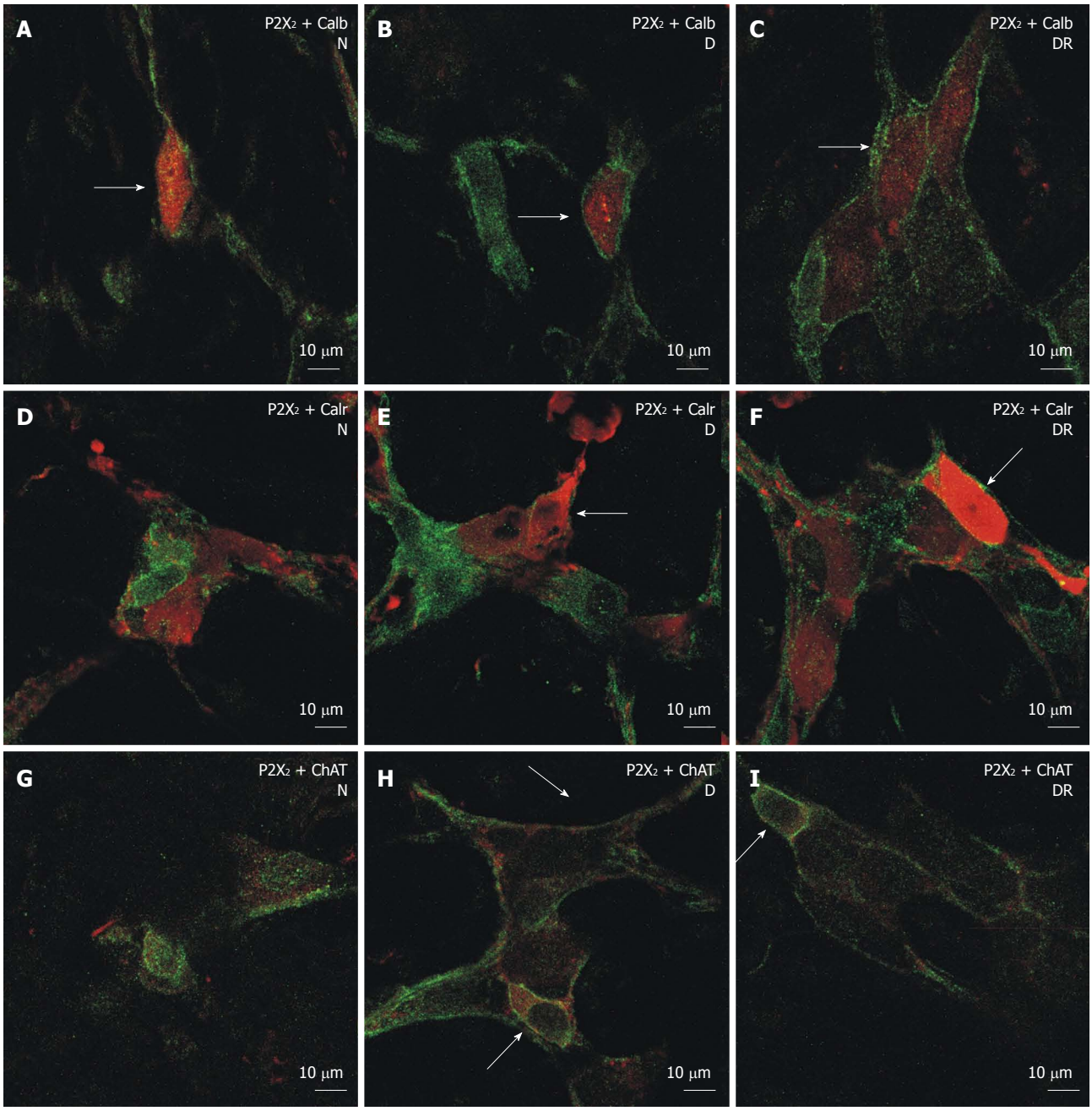

Figure 2 Co-localization of P2X2 receptor immunoreactivity with calbindin, calretinin and choline acetyltransferase immunoreactivity in the ileal submucosal plexus in N, D and DR groups. A-C: P2X2 receptor (green) co-localized with calbindin (Calb) (red); D-F: P2X2 receptor (green) co-localized with calretinin (Calr) (red); G-I: P2X2 receptor (green) co-localized with choline acetyltransferase (ChAT) (red). Double-labeled neurons are indicated by arrows.

IR and ChAT-IR neurons also were immunoreactive for the P2X2 receptor. In the submucosal plexus of the ileum, P2X2-IR neurons were also calbindin-IR, calretinin-IR, and ChAT-IR.

In the myenteric plexus, the majority of NOS-IR neurons were immunoreactive for the P2X2 receptor (N group was 99% ± 0.6% co-localized, D group was 100%, and DR group was 99% ± 0.4%). Also, the majority of calbindin-IR neurons were IR for the P2X2 receptor (N group was 98% ± 0.4%, D group was 100%, and DR group was 99% ± 1%). The majority of calretinin-IR neurons were also IR for the P2X2 receptor (group N was 100%, D group was 98% ± 0.6%, and DR group was 98% ± 1%). Most ChAT-IR neurons were also IR for the P2X2 receptor in the N, D and DR groups (96.2% ± 2%, 96.2% ± 2%, and 97% ± 3%, respectively).

In the submucosal plexus, co-localization between calbindin-IR and P2X2 receptor-IR neurons was complete in the N, D and DR groups. The co-localization between P2X2 receptor-IR and calbindin-IR was 16% ± 0.7% in the N group, 31% ± 2% in the D group, and 24% ± 3% in the DR group ($P < 0.002$). In all three groups, calretinin-IR and ChAT-IR neurons co-localized 100% with P2X2 receptor-IR neurons.

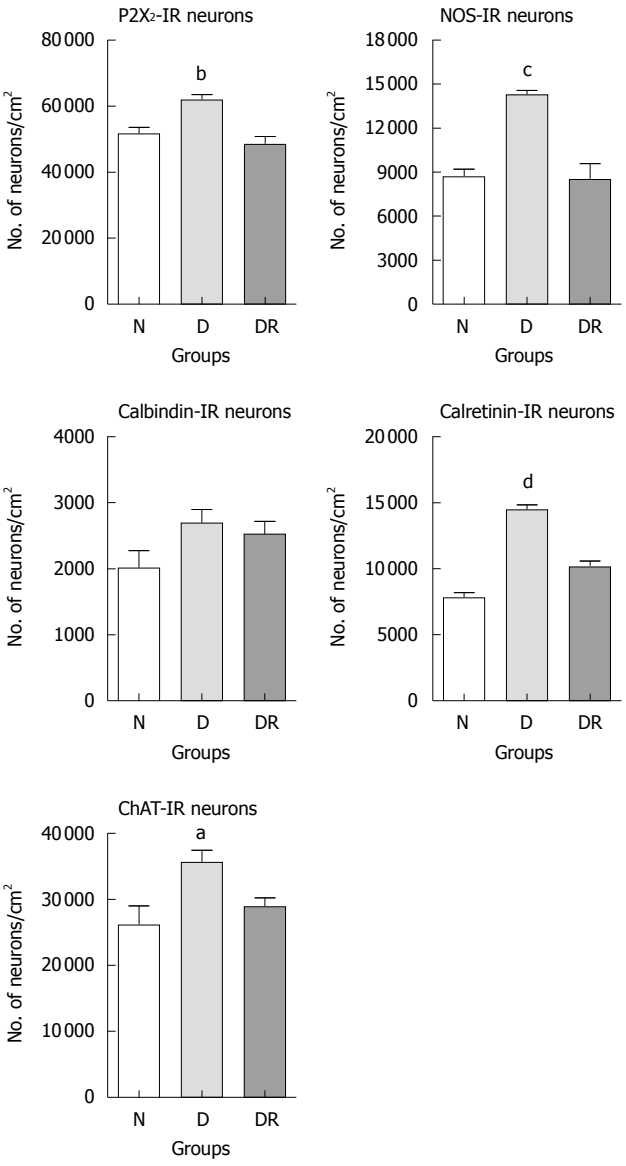

Figure 3 The density (neuron/cm^2) of neurons immunoreactive for the P2X$_2$ receptor, nitric oxide synthase, calbindin, calretinin, and choline acetyltransferase in the ileal myenteric plexus in the N, D and DR groups. $^aP < 0.02$, $^bP < 0.01$, $^cP < 0.002$, $^dP < 0.001$ *vs* N and DR groups. NOS: Nitric oxide synthase; ChAT: Choline acetyltransferase.

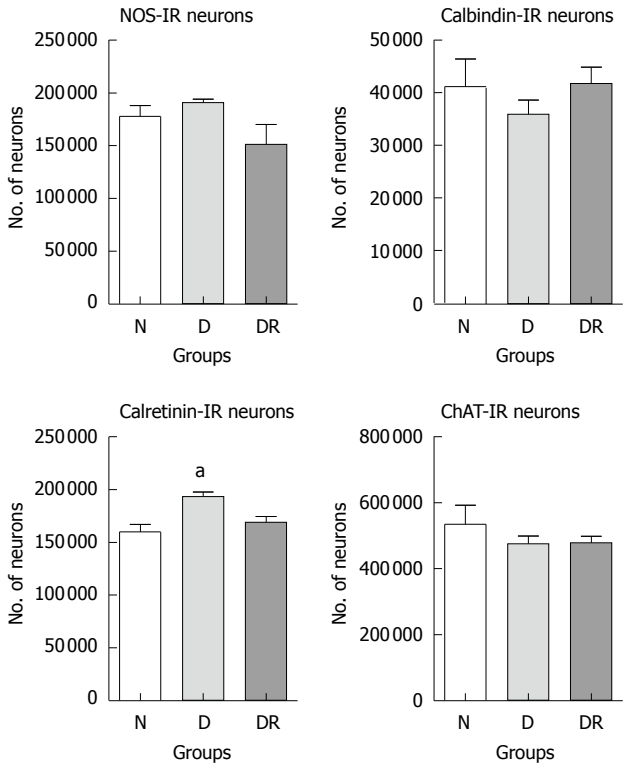

Figure 4 Calculated numbers of neurons immunoreactive for the P2X$_2$ receptor, nitric oxide synthase, calbindin, calretinin and choline acetyltransferase in the small intestine of the N, D and DR groups. $^aP < 0.05$ *vs* N and DR groups. NOS: Nitric oxide synthase; ChAT: Choline acetyltransferase.

Neuronal density

In the myenteric plexus, the number of neurons per unit area was increased by 19% for P2X$_2$ receptor-IR neurons ($P < 0.01$), 64% for NOS-IR neurons ($P < 0.002$), 84% for calretinin-IR neurons ($P < 0.001$), and 26% for ChAT-IR neurons in group D ($P < 0.02$); calbindin-IR neuron density, however, did not differ among the three groups ($P > 0.05$, Figure 3). In the myenteric plexus, the total number of NOS-IR neurons, taking into account the change in intestinal surface area (Figure 4), calbindin-IR neurons and ChAT-IR neurons did not differ significantly between the three groups. There was, however, a 20% increase in the numbers of calretinin-IR neurons and decrease in P2X$_2$ receptor cells with undernutrition relative to controls ($P < 0.05$, Figure 4).

In the submucosal plexus, the density of P2X$_2$ receptor-IR, calbindin-IR and ChAT-IR neurons increased significantly in the undernourished group (67%, $P < 0.0003$; 189%, $P < 0.001$ and 42%, $P < 0.01$, Figure 5). Calretinin-IR neuron density did not differ among the three groups ($P > 0.05$, Figure 5). In the submucosal plexus, the total numbers of the calretinin-IR neurons decreased by 23% ($P < 0.05$), and this was accompanied by an 89% increase in the calculated numbers of calbindin-IR neurons. In this region, there was no change in the numbers of ChAT-IR neurons (Figure 6).

Nerve cell perikarya

Neuron size (nerve cell perikarya, the major and minor axes of the myenteric plexus neurons) of the calretinin-IR neurons were approximately 34% smaller in the protein-deprived rats ($P < 0.001$) than the control or re-fed rats. There was an increase of 35% in the nerve cell perikarya of calbindin-IR neurons and a 14% increase in the minor axes of the calbindin-IR neurons in the DR group, as well as a decrease of 15% in the major axes of the NOS-IR neurons (Table 3).

In the submucosal neurons, there were group differences ($P < 0.05$) with respect to the neuron size of calbindin-IR, calretinin-IR and ChAT-IR neurons. There was a 13% decrease in the major axes of calretinin-IR and ChAT-IR neurons ($P < 0.05$) and an 18% increase in the minor axes of calbindin-IR neurons ($P < 0.05$). Neu-

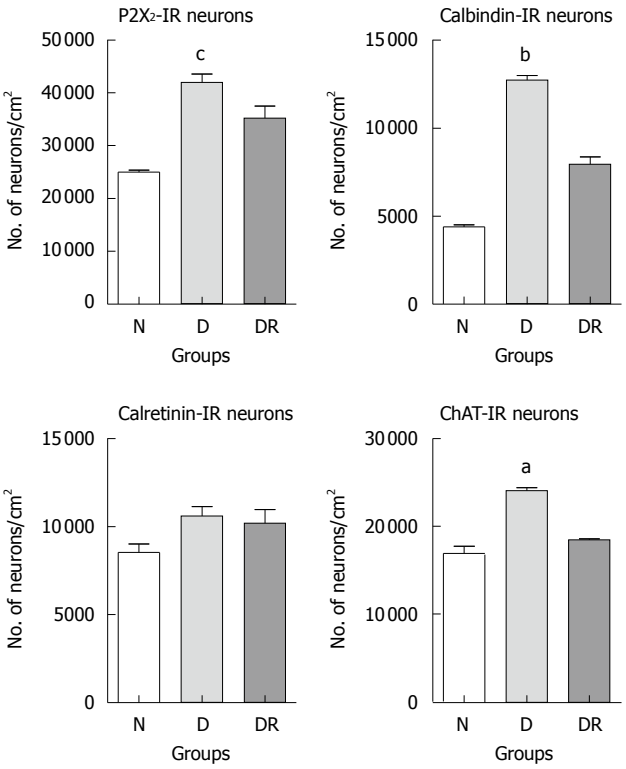

Figure 5 The density of neurons (neuron/cm^2) immunoreactive for P2X$_2$ receptor, calbindin, calretinin and choline acetyltransferase in the ileal submucosal plexus in the N, D and DR groups. $^aP < 0.01$, $^bP < 0.001$, $^cP < 0.0003$ *vs* N and DR groups. ChAT: Choline acetyltransferase.

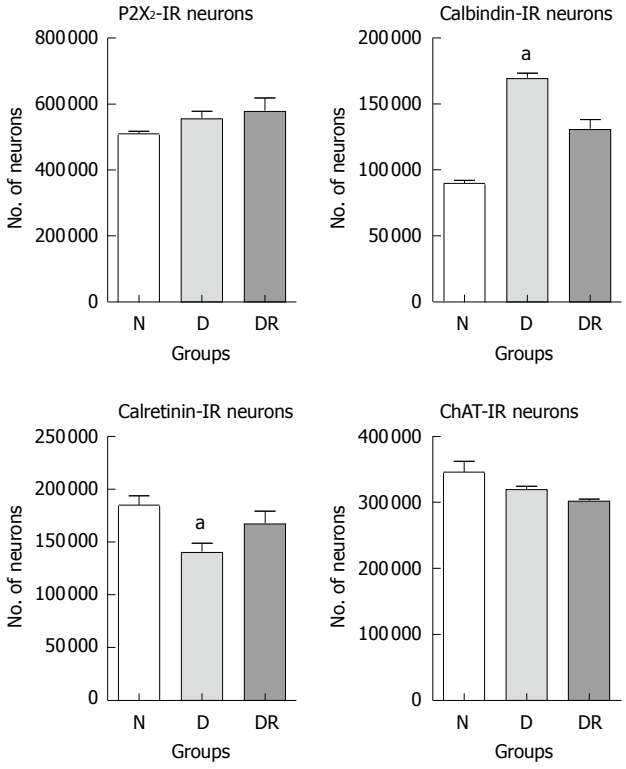

Figure 6 The calculated numbers of neurons immunoreactive for P2X$_2$ receptor, calbindin, calretinin and choline acetyltransferase in the ileal submucosal plexus of the N, D and DR groups. $^aP < 0.05$ *vs* N and DR groups. ChAT: Choline acetyltransferase.

Table 3 Results of the profile area (μm^2), major and minor axes of nitric oxide synthase-IR, calbindin-IR, calretinin-IR and choline acetyltransferase-IR neurons in the ileal myenteric and submucosal plexuses of the N, D and DR groups

	N	D	DR
Myenteric plexus			
NOS			
Profile	240.4 ± 30.7	197.1 ± 25.8	225.8 ± 26.7
Major axes	25.2 ± 2.1	21.7 ± 1.6^a	25.1 ± 1.9
Minor axes	12.1 ± 0.6	11.3 ± 0.8	11.4 ± 1.1
Calbindin			
Profile	227.7 ± 43.1	223.6 ± 26.3	307.5 ± 58^c
Major axes	23.5 ± 2.8	24.1 ± 2.9	27.9 ± 4.6
Minor axes	12.2 ± 1.1	11.9 ± 0.3	14.0 ± 0.8^c
Calretinin			
Profile	397.7 ± 39.6	259.2 ± 48.8^a	331.5 ± 24.5
Major axes	29.3 ± 2.1	22.3 ± 3.5^a	27.5 ± 2.7
Minor axes	16.7 ± 0.6	14.1 ± 0.5^a	15.1 ± 0.5
ChAT			
Profile	229.4 ± 39.4	183.6 ± 39.3	198.7 ± 37.5
Major axes	21.8 ± 2.4	19.4 ± 2.4	20.1 ± 2.5
Minor axes	12.8 ± 1.0	11.7 ± 0.7	12.1 ± 0.8
Submucosal plexus			
Calbindin			
Profile	244.2 ± 47.6	256.7 ± 34.8	310.7 ± 46.2
Major axes	24.8 ± 1.7	27.4 ± 2.2	27.4 ± 1.7
Minor axes	12.4 ± 1.4	12.2 ± 0.8^b	14.5 ± 1.2
Calretinin			
Profile	233.8 ± 51.5	200 ± 4.6	242.5 ± 41.6
Major axes	24.1 ± 2.1	20.8 ± 0.5^b	25.7 ± 3.1
Minor axes	12.4 ± 1.7	12.1 ± 0.3	12.1 ± 1.4
ChAT			
Profile	185.5 ± 18.4	154.3 ± 22.4	175.3 ± 20.0
Major axes	20.1 ± 1.7	17.5 ± 0.8^b	18.8 ± 0.7
Minor axes	11.4 ± 0.2	11.1 ± 0.9	11.5 ± 1.1

$^aP < 0.05$, $^bP < 0.001$ *vs* N and DR groups; $^cP < 0.05$ *vs* N and D groups. Tukey's test for multiple values, mean ± SE, $n = 5$. NOS: Nitric oxide synthase; ChAT: Choline acetyltransferase.

ron size distributions in the myenteric and submucous plexuses of the N, D and DR groups are shown in the histograms of Figures 7 and 8.

DISCUSSION

Various methods have been used to induce experimental undernutrition[26]. The protocols of undernutrition and re-feeding employed in this study were effective, because malnourished animals lost weight, which was then recovered by re-feeding. These findings agree with those of other studies that have used similar protocols[21,22].

The antigen markers for different functional classes of neurons have been determined for guinea pig and mouse small intestine, and to a lesser extent in other mammals[27-31]. The expression patterns have been partly described in the rat[32,33]. NOS is expressed in inhibitory motor neurons in all species in the small and large intestine, whereas all other neuron types, such as excitatory motor neurons, interneurons, and intrinsic primary afferent neurons (IPANs) are immunoreactive for ChAT in the mouse and rat myenteric plexus[30,33-37]. Dogiel Type II neurons, which are intrinsic primary afferent neurons in all species

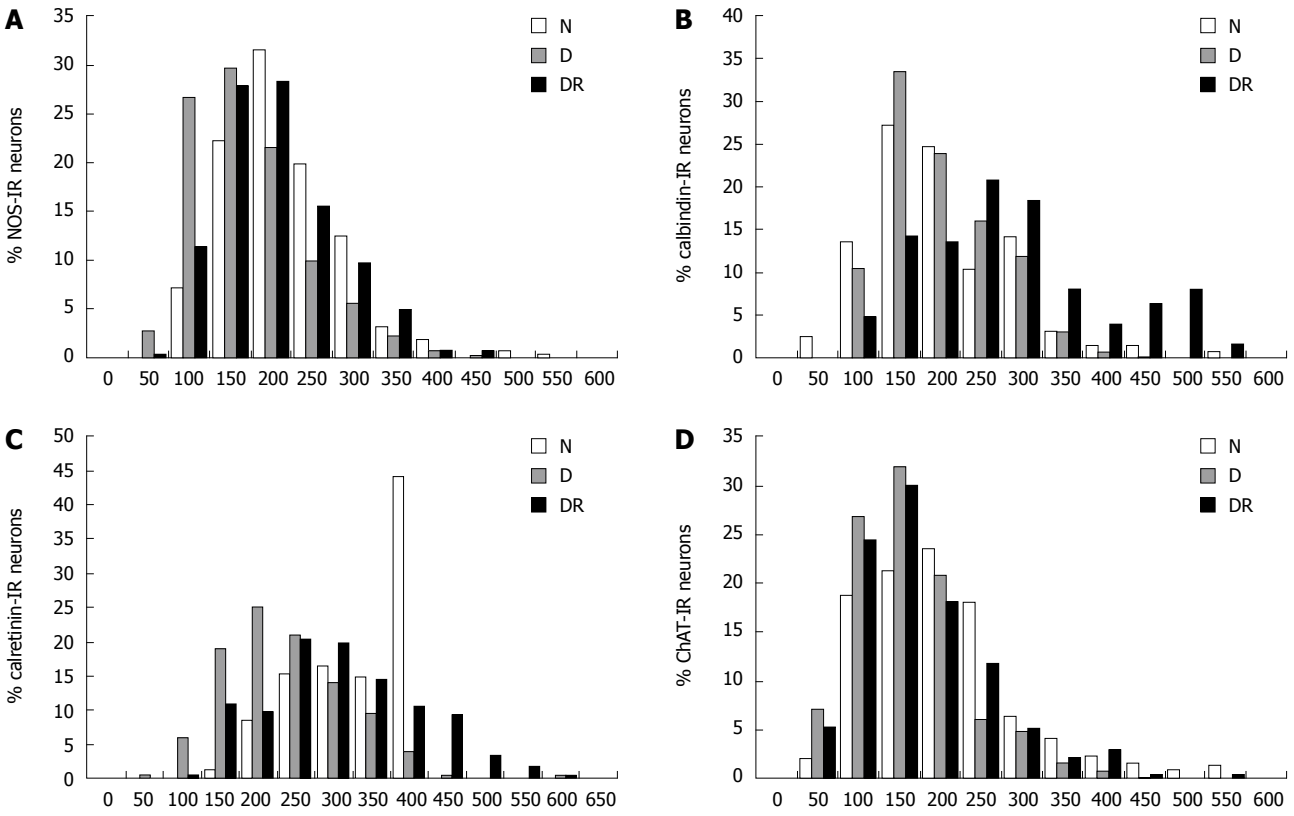

Figure 7 Histograms showing the distribution of areas (μm^2) of neurons immunoreactive for nitric oxide synthase (A), calbindin (B), calretinin (C) and choline acetyltransferase (D) in the ileal myenteric plexus of the N, D and DR groups. NOS: Nitric oxide synthase; ChAT: Choline acetyltransferase.

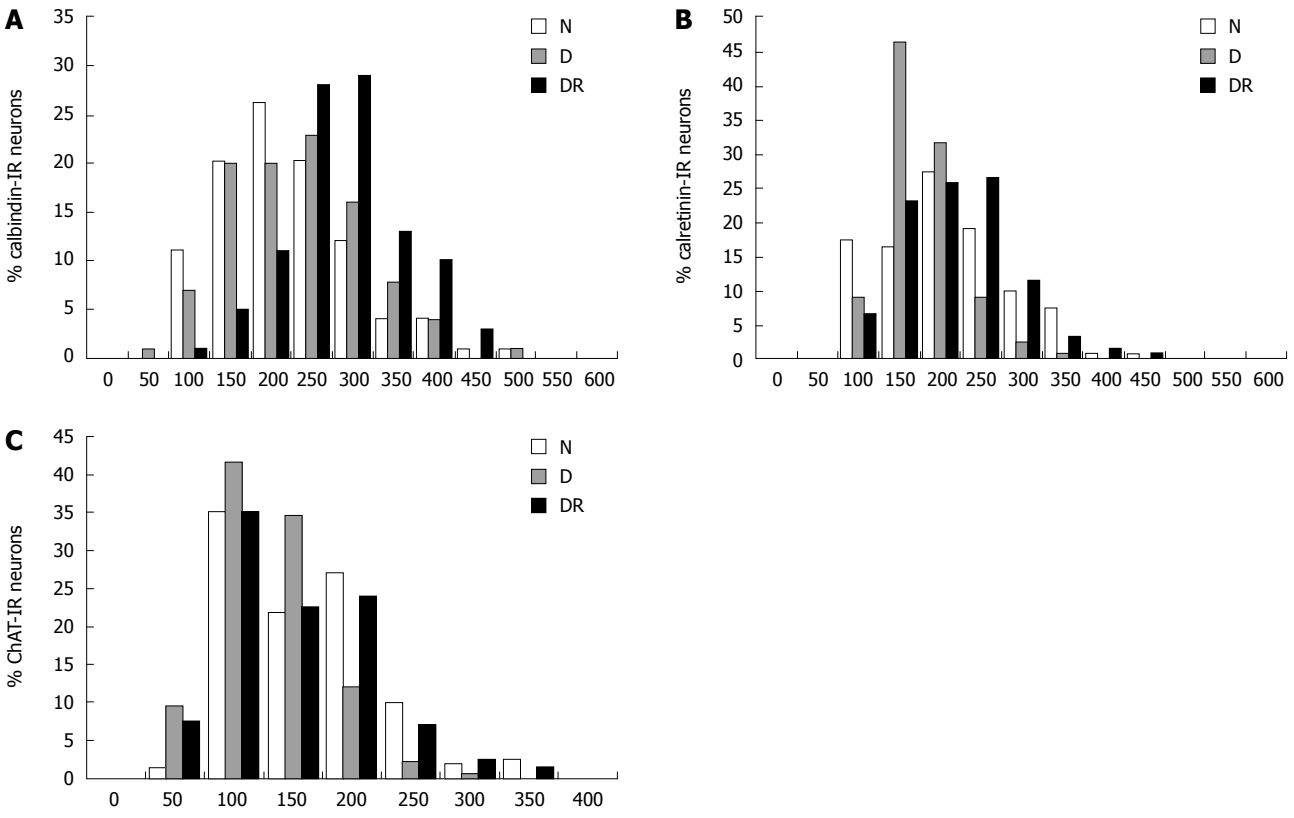

Figure 8 Histogram showing the distribution of areas (μm^2) of neurons immunoreactive for calbindin (A), calretinin (B) and choline acetyltransferase (C) in the ileal submucosal plexus of the N, D and DR groups. ChAT: Choline acetyltransferase.

studied, including rats[38], are immunoreactive for calretinin in the rat small intestine[33]. The subclasses of neurons in the submucosal ganglia of rat ileum have not been extensively studied but, by analogy with other small mammals, they are likely to include cholinergic and non-cholinergic secretomotor neurons and, possibly, IPANs[27,29,37]. In accordance with the data from rats and other small mammals, we chose the enzyme NOS to identify inhibitory motor neurons, ChAT to identify excitatory motor neurons and interneurons, calretinin to identify IPANs, and calbindin, which is a marker of many neurons in the rat small intestine. Within the three groups, the NOS-IR neurons had Dogiel Type I morphology and the calretinin-IR neurons in the myenteric and submucosal plexuses had Dogiel Type Ⅱ morphology while presenting various sizes. Calbindin-IR neurons exhibited four distinct morphologies: the Dogiel type Ⅱ neurons (large and small) and Dogiel type I neurons (small and elongated). These findings are consistent with the literature[37].

By qualitative analyses, there were no differences in neuron morphology between the N, D and DR groups. There was also no observed change in the labeling intensity of neurons immunoreactive for NOS, calretinin, and calbindin among the three groups. However, the intensity of ChAT immunoreactivity was reduced in some neurons of the undernourished group and increased in the re-fed group. These results are consistent with other studies in which a decrease in the intensity of ChAT immunoreactivity in the myenteric neurons of malnourished animals has been reported[22,39]. Other enzymes, such as NADH diaphorase, also show decreased immunoreactivity in malnourished and recovery in re-fed animals[21,22].

Previous studies have revealed the presence of $P2X_2$, $P2X_3$ and $P2X_7$ receptor-containing neurons in the enteric nervous system of guinea pigs[6-11], rats[12-14] and mice[15,16]. In the current work with the rat enteric nervous system, we found that $P2X_2$ receptors were present in both the cytoplasm and cytoplasmic membrane in neurons of the myenteric and submucosal plexuses.

The co-localization of different neuronal markers described in this work confirm the presence of $P2X_2$ receptors in NOS-IR, calretinin-IR, and calbindin-IR enteric neurons, as well as in ChAT-IR neurons of the myenteric and submucosal plexuses. ATP has been reported to depolarize 70%-90% of guinea-pig enteric neurons, which indicates that many enteric neurons have ionic P2X receptors[2,3]. In the myenteric plexus of rats, we found that the $P2X_2$ receptors exhibited complete co-localization with NOS-IR, calretinin-IR, calbindin-IR and ChAT-IR neurons in the three groups examined, without significant differences among them. This finding is consistent with the literature, which demonstrates the presence of the receptor in inhibitory neurons, as well as intrinsic excitatory and secretomotor/vasodilator primary afferent neurons in guinea pigs[7] and rats[12].

Our analyses of co-localization in the submucosal plexus showed that all calretinin-IR, ChAT and calbindin-IR neurons co-localized with $P2X_2$ receptor-IR neurons. However, there was a significant increase ($P < 0.05$) in the co-localization of $P2X_2$ neurons with calbindin-IR neurons in the undernourished group, which recovered in the re-fed DR group. This result agrees with Xiang and Burnstock's[12] findings, in which they reported expression of $P2X_2$ receptor in calretinin and calbindin neurons in the ileal myenteric plexus of rats. The co-localization that we described in the myenteric and submucosal plexuses suggested that malnutrition did not change neurochemical coding, for the markers that were used, in the enteric nervous system.

Changes in the density of myenteric neurons have been observed in various regions of the gastrointestinal tract in models of undernutrition[20-23,25,39] and recovery is observed in re-fed rats[21,22,25]. The increase in neuronal density in undernourished protocols is likely due to decreases in the surface area of the small or large intestine[20-22,39]. In our work on the myenteric plexus, neuron densities were increased for $P2X_2$ receptor-IR, NOS-IR, ChAT-IR and calretinin-IR neurons in the D group, and went back to control levels in the DR group. This increase in neuron density was due to a reduction of approximately 34% in small-intestinal area in the D group. There was recovery of the intestinal area in the DR group. In contrast, the density of calbindin-IR neurons in the myenteric plexus did not differ among the three groups ($P > 0.05$). Moreover, the increases in neuron density in the myenteric plexus in the D group were dependent upon the neuronal class examined. NOS-IR neuron density increased by 64%, calretinin-IR neurons by 84%, and ChAT-IR neurons by 26%; these data suggest that undernourishment affects the neuronal subtypes differently. There was no change in the calculation of the total number of NOS-IR, calbindin-IR or ChAT-IR neurons in the small intestine of the three groups. However, the calretinin-IR neuron numbers were increased (20%) in the undernourished group and $P2X_2$ receptor-IR neurons were decreased by around 25% in the D and DR groups.

The density of $P2X_2$-receptor-expressing neurons in the myenteric plexus in group N was about 51 000/cm^2 in our study. This value is higher than the combined sum of the two major neuronal subtype populations of the myenteric neurons: NOS (8000/cm^2) + ChAT (26 000/cm^2). This discrepancy could be due to $P2X_2$ receptor staining in another neuronal class, which was not immunoreactive for NOS, calbindin, calretinin or ChAT. Also, $P2X_2$ receptor labeling could have also stained enteric glial cells. The presence of P2X and P2Y receptors has been described in astrocytes and microglia of the central nervous system[40,41] and in enteric glial cells[9,42]. In the mammalian enteric nervous system, the proportion of glial cells to neurons is about three to one[43-45].

The tonic release of ATP into the extracellular space without a particular stimulus is a widespread physiological process. However, the release of ATP into the extracellular environment is also caused by pathophysiological events like inflammation, ischemia, injury as a consequence of cell damage or acute cell death, and metabolic

stress[46]. All physiological effects of ATP including fast purinergic transmission and co-transmission, the secretion of neuropeptides, and mechanosensory transduction might be amplified by overtly increased extracellular concentrations of ATP[46].

Studies from the literature have reported changes in the expression of purinergic receptors in different dietary conditions in the central nervous system. A diet deficient in zinc, for example, increases expression of $P2X_6$ receptors in the hippocampus of rats[47], which suggests that dietary zinc levels also affect protein expression and could act as a modulator of the receptor function. Increased $P2Y_1$ receptor mRNA expression in the hypothalamus after food restriction has been reported in rats[48], and the data indicate that expression of ADP/ATP-sensitive $P2Y_1$ receptors in the hypothalamus is dependent on feeding conditions. The enhanced expression of the $P2Y_1$ receptor during the early and late interval of restricted feeding suggests an increased demand for purinergic signaling to enhance the activity of hypothalamic neurons. Also, there is an indication of $P2Y_1$ and A_{2A} that purinergic receptor mRNA expression is altered during acute and chronic food deprivation[49]. Some authors have suggested that ATP/ADP, acting as extracellular signal molecules in the rat brain, is involved in the regulation of food intake, possibly depending on $P2Y_1$-receptor-mediated nitric oxide production[50].

During metabolic stress, such as hypoglycemia or brain ischemia, activation of different P2 receptors has been demonstrated *in vivo* and *in vitro*. The $P2X_2$ and $P2X_4$ receptors are upregulated after oxygen and glucose deprivation in organotypical slice cultures and in CA1 and CA3 pyramidal cells after *in vivo* ischemia in gerbils[51]. During *in vivo* and *in vitro* ischemia, the $P2X_7$ receptor density is upregulated in microglia and on astrocytes and neurons[52]. Prenatal protein malnutrition might increase circulating concentrations of ATP, and this increases $P2X_2$ expression in cells. Enhancement of $P2X_2$ receptors in the D group suggests an increased demand for purinergic signaling. These changes were all reversed in re-fed rats, which demonstrated the effectiveness of re-feeding upon enteric neuron recovery.

Changes in neuronal expression of $P2X_{1-7}$ purinoceptors are frequently seen not only as a result of maturation and neuronal differentiation, but also after various types of acute insults to the central nervous system such as ischemia, hypoxia, mechanical stress, axotomy, and inflammation. Purinergic mechanisms are involved in the etiology of many neurodegenerative conditions, especially due to the large extracellular release of ATP, adenosine, and other neurotransmitters[46,53] upon neural damage. Prolonged stimulation of ATP receptors results in changes in the location and density of P2 receptors in the cell membrane[46]. Increased $P2X_3$ receptor expression has been observed in inflammatory bowel disease of the large intestine, which suggests that changes in this receptor can cause pain and dysmotility of the bowel[54].

Nitrergic neurons (using nitric oxide synthesized by NOS) and cholinergic neurons (those that use the acetylcholine synthesized by ChAT) represent two major subpopulations of myenteric neurons[55], although these patterns vary between guinea pigs[56], mice[30,57] and rats[33,36].

Our work implies that differences between groups in the total neuronal density in the myenteric plexus are comprised principally of changes in the NOS-IR-and ChAT-IR neuronal populations. The total neuron density in the myenteric plexus was approximately 34 800/cm^2 in the nourished group. This neuronal density is greater in comparison with that in previous studies, which have reported values of 15 000 to 20 000/cm^2[58], 10 000/cm^2[59] and 18 000/cm^2[60]. These differences could be, in part, due to methodology as well as the different ages and strains of rats used.

Marese *et al*[61] have quantified neuronal numbers in the myenteric plexus of the duodenum using the Giemsa histological method and myosin V pan-neuronal immunohistochemical labeling. These studies have demonstrated that the number of neurons/cm^2 decreases with animal age between 21 and 428 d. Our experiments used 42-d-old rats of the same lineage (Wistar), and the estimate of neuron density in our work is within the 21-60 d range reported by Marese *et al*[61] (21 d: Giemsa 89 335 neurons/cm^2; myosin V: 59 364/cm^2; 60 d: Giemsa: 47 814/cm^2; myosin V: 30 291/cm^2).

In the present work, the proportions of NOS-IR and ChAT-IR neurons were 32% and 68%, respectively, in the myenteric plexus of the malnourished group. Consistent with previous studies[35,56], we found that these proportions were maintained in the D and DR groups.

Submucosal plexus

ChAT-IR neurons comprised the majority of submucosal plexus neurons in the three groups. These findings agree with prior studies, which have described most neurons of the submucosal plexus as ChAT-IR[33,56]. We demonstrated, for the first time, an increase in the density of $P2X_2$ receptor-IR, calbindin-IR, calretinin-IR and ChAT-IR neurons in the deprived animals, which returned to control levels in the re-fed animals in the submucosal plexus. This increase was due to a 34% reduction in the area of the small intestine in the deprived animals.

The total number of calbindin-IR submucosal neurons increased in the small intestine of undernourished animals, in contrast to a decrease in calretinin-IR neurons and no change in the number of ChAT-IR neurons. These data indicate that the lack of protein nutrition can also have an impact on the chemical coding of the submucosal plexus. Differences in these measures between the myenteric and submucosal plexuses might reflect a differential effect of malnutrition or undernutrition on these two regions, as well as a differential effect of undernourishment on each neuronal subtype. In addition, the increase of calbindin-expressing neurons in the submucosal plexus could be a compensatory mechanism in response to the decrease in these neurons in the myenteric plexus.

Neuronal sizes

Previous immunohistochemical studies have shown that

undernutrition affects the neuron size profile of the gastrointestinal tract[20,21,39]. Analyses using the Giemsa technique[20,62] and histochemistry[22] have found no significant differences in the neuronal sizes in the small intestine in nourished, undernourished and re-fed animals. The present work, using an immunohistochemistry technique, was unable to verify exactly which neuronal class showed changes in size. In the myenteric plexus, there were decreases in size of the calretinin-IR neurons in groups D and DR. There was also an increase in the size of calbindin-IR neurons in the DR group, compared to the N and D groups. There was no change ($P > 0.05$) in the size of ChAT-IR neurons among the three groups. The size of NOS-IR neurons also did not change, consistent with previous reports[58]. In the submucosal plexus, the sizes of calbindin-IR, calretinin-IR and ChAT-IR neurons were not affected by undernutrition. However, the major axes of the calbindin-IR and minor axes of the calretinin-IR and ChAT-IR neurons decreased in group D, with recovery in group DR. The differences between the submucosal and myenteric plexuses suggest again that undernutrition affects the two plexuses differently. The distribution areas of NOS-IR, calbindin-IR, calretinin-IR and ChAT-IR neurons in our study ranged from 100 to 500 μm^2, in agreement with previous reports[22].

The current study demonstrates that both undernourishment and re-feeding has a different impact on neuronal subtypes. Undernutrition also differently affects the myenteric and submucosal plexuses; changes in calbindin-IR neuronal density in the submucosal plexus were not reflected in the myenteric plexus, where only the profile of the calretinin-IR neurons was affected by dietary restriction. These changes were all reversed in re-fed rats, which demonstrated the effectiveness of re-feeding upon enteric neuron recovery.

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COMMENTS

Background

The myenteric and submucous plexuses are affected in undernourished and re-fed rats.

Research frontiers

The chemical coding and $P2X_2$ receptor expression following malnourishment and re-feeding are unknown.

Innovations and breakthroughs

The present study showed the effects of undernourishment and re-feeding on the morphology of the $P2X_2$-immunoreactive (IR), nitric oxide synthase-IR, calbindin-IR, calretinin-IR and choline acetyltransferase-IR neurons of the myenteric and submucosal plexuses.

Applications

The present study suggests that re-feeding can restore almost all of the changes to inhibitory intrinsic primary afferent neurons and cholinergic neurons seen in undernourished animals.

Peer review

This is a study of the effects of deprivation and restoration of protein on neuronal development in the submucosal and myenteric plexuses. The results describe changes induced by protein deprivation that are largely reversible by re-feeding.

REFERENCES

1 **Burnstock G**. The past, present and future of purine nucleotides as signalling molecules. *Neuropharmacology* 1997; **36**: 1127-1139
2 **Barajas-López C**, Huizinga JD, Collins SM, Gerzanich V, Espinosa-Luna R, Peres AL. P2x-purinoceptors of myenteric neurones from the guinea-pig ileum and their unusual pharmacological properties. *Br J Pharmacol* 1996; **119**: 1541-1548
3 **Bian X**, Bertrand PP, Bornstein JC. Descending inhibitory reflexes involve P2X receptor-mediated transmission from interneurons to motor neurons in guinea-pig ileum. *J Physiol* 2000; **528**: 551-560
4 **Galligan JJ**, LePard KJ, Schneider DA, Zhou X. Multiple mechanisms of fast excitatory synaptic transmission in the enteric nervous system. *J Auton Nerv Syst* 2000; **81**: 97-103
5 **Galligan JJ**. Ligand-gated ion channels in the enteric nervous system. *Neurogastroenterol Motil* 2002; **14**: 611-623
6 **Vulchanova L**, Arvidsson U, Riedl M, Wang J, Buell G, Surprenant A, North RA, Elde R. Differential distribution of two ATP-gated channels (P2X receptors) determined by immunocytochemistry. *Proc Natl Acad Sci USA* 1996; **93**: 8063-8067
7 **Castelucci P**, Robbins HL, Poole DP, Furness JB. The distribution of purine P2X(2) receptors in the guinea-pig enteric nervous system. *Histochem Cell Biol* 2002; **117**: 415-422
8 **Poole DP**, Castelucci P, Robbins HL, Chiocchetti R, Furness JB. The distribution of P2X3 purine receptor subunits in the guinea pig enteric nervous system. *Auton Neurosci* 2002; **101**: 39-47
9 **Van Nassauw L**, Brouns I, Adriaensen D, Burnstock G, Timmermans JP. Neurochemical identification of enteric neurons expressing P2X(3) receptors in the guinea-pig ileum. *Histochem Cell Biol* 2002; **118**: 193-203
10 **Hu HZ**, Gao N, Lin Z, Gao C, Liu S, Ren J, Xia Y, Wood JD. P2X(7) receptors in the enteric nervous system of guinea-pig small intestine. *J Comp Neurol* 2001; **440**: 299-310
11 **Xiang Z**, Burnstock G. Distribution of P2Y2 receptors in the guinea pig enteric nervous system and its coexistence with P2X2 and P2X3 receptors, neuropeptide Y, nitric oxide synthase and calretinin. *Histochem Cell Biol* 2005; **124**: 379-390
12 **Xiang Z**, Burnstock G. P2X2 and P2X3 purinoceptors in the rat enteric nervous system. *Histochem Cell Biol* 2004; **121**: 169-179
13 **Xiang Z**, Burnstock G. Development of nerves expressing P2X3 receptors in the myenteric plexus of rat stomach. *Histochem Cell Biol* 2004; **122**: 111-119
14 **Yu Q**, Zhao Z, Sun J, Guo W, Fu J, Burnstock G, He C, Xiang Z. Expression of P2X6 receptors in the enteric nervous system of the rat gastrointestinal tract. *Histochem Cell Biol* 2010; **133**: 177-188
15 **Giaroni C**, Knight GE, Ruan HZ, Glass R, Bardini M, Lecchini S, Frigo G, Burnstock G. P2 receptors in the murine gastrointestinal tract. *Neuropharmacology* 2002; **43**: 1313-1323
16 **Ruan HZ**, Burnstock G. The distribution of P2X5 purinergic receptors in the enteric nervous system of mouse. *Cell Tissue Res* 2005; **319**: 191-200
17 **Cornblath DR**, Brown MJ. Influence of malnutrition on developing rat peripheral nerves. *Exp Neurol* 1988; **99**: 403-411
18 **Santer RM**, Conboy VB. Prenatal undernutrition permanently decreases enteric neuron number and sympathetic innervation of Auerbach's plexus in the rat. *J Anat* 1990; **168**: 57-62
19 **Leite-Mello EV**, Stabille SR, Miranda Neto MH. Effect of maternal protein deprivation on morphological and quanti-

tative aspects of the myenteric plexus neurons of proximal colon in rats. *Arq Neuropsiquiatr* 1997; **55**: 106-113
20 **Sant'ana Dde M**, Miranda Neto MH, de Souza RR, Molinari SL. Morphological and quantitative study of the myenteric plexus of the ascending colon of rats subjected to proteic desnutrition. *Arq Neuropsiquiatr* 1997; **55**: 687-695
21 **Castelucci P**, de Souza RR, de Angelis RC, Furness JB, Liberti EA. Effects of pre- and postnatal protein deprivation and postnatal refeeding on myenteric neurons of the rat large intestine: a quantitative morphological study. *Cell Tissue Res* 2002; **310**: 1-7
22 **Gomes OA**, Castelucci P, de Vasconcellos Fontes RB, Liberti EA. Effects of pre- and postnatal protein deprivation and postnatal refeeding on myenteric neurons of the rat small intestine: a quantitative morphological study. *Auton Neurosci* 2006; **126-127**: 277-284
23 **Liberti EA**, Fontes RB, Fuggi VM, Maifrino LB, Souza RR. Effects of combined pre- and post-natal protein deprivation on the myenteric plexus of the esophagus of weanling rats: a histochemical, quantitative and ultrastructural study. *World J Gastroenterol* 2007; **13**: 3598-3604
24 **Gomes SP**, Nyengaard JR, Misawa R, Girotti PA, Castelucci P, Blazquez FH, de Melo MP, Ribeiro AA. Atrophy and neuron loss: effects of a protein-deficient diet on sympathetic neurons. *J Neurosci Res* 2009; **87**: 3568-3575
25 **Greggio FM**, Fontes RB, Maifrino LB, Castelucci P, de Souza RR, Liberti EA. Effects of perinatal protein deprivation and recovery on esophageal myenteric plexus. *World J Gastroenterol* 2010; **16**: 563-570
26 **Cha CJ**, Gelardi NL, Oh W. Growth and cellular composition in rats with intrauterine growth retardation: effects of postnatal nutrition. *J Nutr* 1987; **117**: 1463-1468
27 **Furness JB**. Types of neurons in the enteric nervous system. *J Auton Nerv Syst* 2000; **81**: 87-96
28 **Brehmer A**. Structure of enteric neurons. *Adv Anat Embryol Cell Biol* 2006; **186**: 1-91
29 **Furness JB**. The Enteric Nervous System. Malden: Blackwell Publishing Inc., 2006
30 **Qu ZD**, Thacker M, Castelucci P, Bagyánszki M, Epstein ML, Furness JB. Immunohistochemical analysis of neuron types in the mouse small intestine. *Cell Tissue Res* 2008; **334**: 147-161
31 **Mongardi Fantaguzzi C**, Thacker M, Chiocchetti R, Furness JB. Identification of neuron types in the submucosal ganglia of the mouse ileum. *Cell Tissue Res* 2009; **336**: 179-189
32 **Ekblad E**, Mulder H, Uddman R, Sundler F. NOS-containing neurons in the rat gut and coeliac ganglia. *Neuropharmacology* 1994; **33**: 1323-1331
33 **Mann PT**, Furness JB, Southwell BR. Choline acetyltransferase immunoreactivity of putative intrinsic primary afferent neurons in the rat ileum. *Cell Tissue Res* 1999; **297**: 241-248
34 **Aimi Y**, Kimura H, Kinoshita T, Minami Y, Fujimura M, Vincent SR. Histochemical localization of nitric oxide synthase in rat enteric nervous system. *Neuroscience* 1993; **53**: 553-560
35 **Jarvinen MK**, Wollmann WJ, Powrozek TA, Schultz JA, Powley TL. Nitric oxide synthase-containing neurons in the myenteric plexus of the rat gastrointestinal tract: distribution and regional density. *Anat Embryol* (Berl) 1999; **199**: 99-112
36 **Nakajima K**, Tooyama I, Yasuhara O, Aimi Y, Kimura H. Immunohistochemical demonstration of choline acetyltransferase of a peripheral type (pChAT) in the enteric nervous system of rats. *J Chem Neuroanat* 2000; **18**: 31-40
37 **Sayegh AI**, Ritter RC. Morphology and distribution of nitric oxide synthase-, neurokinin-1 receptor-, calretinin-, calbindin-, and neurofilament-M-immunoreactive neurons in the myenteric and submucosal plexuses of the rat small intestine. *Anat Rec A Discov Mol Cell Evol Biol* 2003; **271**: 209-216
38 **Kunze WA**, Mao YK, Wang B, Huizinga JD, Ma X, Forsythe P, Bienenstock J. Lactobacillus reuteri enhances excitability of colonic AH neurons by inhibiting calcium-dependent potassium channel opening. *J Cell Mol Med* 2009; **13**: 2261-2270
39 **Brandão MCS**, De Angelis RC, De-Souza RR, Fróes LB, Liberti EA. Effects of pre- and postnatal protein energy deprivation on the myenteric plexus of the small intestine: a morphometric study in weanling rats. *Nutrition Res* 2003; **23**: 215-223
40 **Lalo U**, Pankratov Y, Wichert SP, Rossner MJ, North RA, Kirchhoff F, Verkhratsky A. P2X1 and P2X5 subunits form the functional P2X receptor in mouse cortical astrocytes. *J Neurosci* 2008; **28**: 5473-5480
41 **Abbracchio MP**, Burnstock G, Verkhratsky A, Zimmermann H. Purinergic signalling in the nervous system: an overview. *Trends Neurosci* 2009; **32**: 19-29
42 **Vanderwinden JM**, Timmermans JP, Schiffmann SN. Glial cells, but not interstitial cells, express P2X7, an ionotropic purinergic receptor, in rat gastrointestinal musculature. *Cell Tissue Res* 2003; **312**: 149-154
43 **Gabella G**, Trigg P. Size of neurons and glial cells in the enteric ganglia of mice, guinea-pigs, rabbits and sheep. *J Neurocytol* 1984; **13**: 49-71
44 **Rühl A**. Glial cells in the gut. *Neurogastroenterol Motil* 2005; **17**: 777-790
45 **Hoff S**, Zeller F, von Weyhern CW, Wegner M, Schemann M, Michel K, Rühl A. Quantitative assessment of glial cells in the human and guinea pig enteric nervous system with an anti-Sox8/9/10 antibody. *J Comp Neurol* 2008; **509**: 356-371
46 **Franke H**, Krügel U, Illes P. P2 receptors and neuronal injury. *Pflugers Arch* 2006; **452**: 622-644
47 **Chu Y**, Mouat MF, Coffield JA, Orlando R, Grider A. Expression of P2X6, a purinergic receptor subunit, is affected by dietary zinc deficiency in rat hippocampus. *Biol Trace Elem Res* 2003; **91**: 77-87
48 **Seidel B**, Bigl M, Franke H, Kittner H, Kiess W, Illes P, Krügel U. Expression of purinergic receptors in the hypothalamus of the rat is modified by reduced food availability. *Brain Res* 2006; **1089**: 143-152
49 **Krügel U**, Seidel B, Spies O, Kitter H, Illes P, Kiess W. Chronic food restriction alters purinergic receptor mRNA expression in the nucleus accumbens of the rat. *Drug Devel Res* 2003; **59**: 95-103
50 **Kittner H**, Franke H, Harsch JI, El-Ashmawy IM, Seidel B, Krügel U, Illes P. Enhanced food intake after stimulation of hypothalamic P2Y1 receptors in rats: modulation of feeding behaviour by extracellular nucleotides. *Eur J Neurosci* 2006; **24**: 2049-2056
51 **Cavaliere F**, Florenzano F, Amadio S, Fusco FR, Viscomi MT, D'Ambrosi N, Vacca F, Sancesario G, Bernardi G, Molinari M, Volontè C. Up-regulation of P2X2, P2X4 receptor and ischemic cell death: prevention by P2 antagonists. *Neuroscience* 2003; **120**: 85-98
52 **Franke H**, Günther A, Grosche J, Schmidt R, Rossner S, Reinhardt R, Faber-Zuschratter H, Schneider D, Illes P. P2X7 receptor expression after ischemia in the cerebral cortex of rats. *J Neuropathol Exp Neurol* 2004; **63**: 686-699
53 **Franke H**, Illes P. Involvement of P2 receptors in the growth and survival of neurons in the CNS. *Pharmacol Ther* 2006; **109**: 297-324
54 **Yiangou Y**, Facer P, Baecker PA, Ford AP, Knowles CH, Chan CL, Williams NS, Anand P. ATP-gated ion channel P2X(3) is increased in human inflammatory bowel disease. *Neurogastroenterol Motil* 2001; **13**: 365-369
55 **Phillips RJ**, Kieffer EJ, Powley TL. Aging of the myenteric plexus: neuronal loss is specific to cholinergic neurons. *Auton Neurosci* 2003; **106**: 69-83
56 **Chiocchetti R**, Poole DP, Kimura H, Aimi Y, Robbins HL, Castelucci P, Furness JB. Evidence that two forms of choline

acetyltransferase are differentially expressed in subclasses of enteric neurons. *Cell Tissue Res* 2003; **311**: 11-22
57 **Sang Q**, Young HM. Chemical coding of neurons in the myenteric plexus and external muscle of the small and large intestine of the mouse. *Cell Tissue Res* 1996; **284**: 39-53
58 **Cowen T**, Johnson RJ, Soubeyre V, Santer RM. Restricted diet rescues rat enteric motor neurones from age related cell death. *Gut* 2000; **47**: 653-660
59 **Heinicke EA**, Kiernan JA, Wijsman J. Specific, selective, and complete staining of neurons of the myenteric plexus, using cuprolinic blue. *J Neurosci Methods* 1987; **21**: 45-54
60 **Phillips RJ**, Hargrave SL, Rhodes BS, Zopf DA, Powley TL. Quantification of neurons in the myenteric plexus: an evaluation of putative pan-neuronal markers. *J Neurosci Methods* 2004; **133**: 99-107
61 **Marese AC**, de Freitas P, Natali MR. Alterations of the number and the profile of myenteric neurons of Wistar rats promoted by age. *Auton Neurosci* 2007; **137**: 10-18
62 **Marçal Natali MR**, Molinari SL, Valentini LC, Hubner de Miranda Neto M. Morphoquantitative evaluation of the duodenal myenteric neuronal population in rats fed with hypoproteic ration. *Biocell* 2005; **29**: 39-46

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ORIGINAL ARTICLE

Proteomic analysis reveals molecular biological details in varioliform gastritis without *Helicobacter pylori* infection

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Abstract

AIM: To investigate and elucidate the molecular mechanism underlying varioliform gastritis for early detection, prevention and intervention of gastric cancer.

METHODS: A combination of two-dimensional gel electrophoresis and mass spectrometry was used to detect the differentially expressed proteins between varioliform gastritis and matched normal mucosa. The selected proteins were confirmed by Western blotting and reverse transcription polymerase chain reaction (RT-PCR) in additional samples and the function of some proteins in varioliform gastritis was analyzed by bio-method preliminarily.

RESULTS: We identified 21 differentially expressed proteins in varioliform gastritis, and compared them with matched normal mucosa. Eleven proteins were upregulated and ten downregulated in varioliform gastritis when compared with the same proteins in individual-matched normal gastric mucosa. These proteins are related to metabolism, oxidation, cytoskeleton, apoptosis, signal transduction and other aspects of cells. Two novel proteins, thioredoxin domain-containing protein 5 (TXNDC5) upregulated in varioliform gastritis, and neuropolypeptide h3 [phosphatidylethanolamine-binding protein 1 (PEBP1)] downregulated in varioliform gastritis, were further investigated. Their expressions were validated by Western blotting and RT-PCR in 12 cases of varioliform gastritis which was matched with normal mucosa. The expression level of PEBP1 in varioliform gastritis was significantly lower ($P < 0.05$) while that of TXNDC5 was significantly higher than that in matched normal gastric mucosa ($P < 0.05$).

CONCLUSION: There are some changes of protein expression in varioliform gastritis. Downregulation of PEBP1 and upregulation of TXNDC5 are involved in the development of varioliform gastritis.

Key words: Differentially expressed proteins; Varioliform gastritis; Proteomic study; *Helicobacter pylori*

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INTRODUCTION

Varioliform gastritis is currently recognized as a special kind of chronic gastritis characterized by nodules, thickened fugal folds and erosions. These features appear to be

unusual and different from those seen in chronic gastritis. The diagnosis can be easily made by endoscopic examination. But the morbidity of varioliform gastritis has increased quickly recently in China. Very little is known about the etiopathogeny, clinical significance and evolution of this disease. The molecular biological researches on varioliform gastritis are very limited and no proteomics research on this disease has been found up to date. So the molecular mechanism of this disease is still unclear. The role of *Helicobacter pylori (H. pylori)* remains unknown. Although a close relationship between this gastritis and the bacteria was suggested to exist over the last few years, But no *H. pylori* infection was found in the gastric mucosa of some patients with varioliform gastritis. What is the reason?

Gastric cancer is the second most common malignancy in the world. Each year, about 798 000 people are diagnosed as having gastric cancer (9.9% of total cancer cases) and 628 000 people die from the disease (12.1% of cancer deaths)[1]. In eastern Asian countries including China, the morbidity and mortality of gastric cancer have ranked the first among all kinds of cancer and grown rapidly in the past two decades. Gastric carcinogenesis is not a well-known process, and the central paradigm for the initiation and development of gastric carcinoma is still not very clear.

In 1960, Munoz Monteavaro *et al*[2] reported varioliform gastritis with "*in situ*" carcinomatous transformation. It was reported a case of ampullary carcinoma accompanied with gastroenteropathy due to diffuse varioliform gastritis. Similarly, Cappell *et al*[3] reported adenomatous transformation in a patient with varioliform gastritis who had serial gastroscopies. This report also suggests a possible association between varioliform gastritis and gastric neoplasia. Several other groups have reported similar findings and performed a more comprehensive analysis of relationship between varioliform gastritis and gastric cancer[4-6].

The elevations could persist and appear as sessile polyps after the erosions heal and symptoms relieved after treatment. Adenomatous transformation was reported in some patients with varioliform gastritis. These reports suggested a possible association between varioliform gastritis and gastric neoplasia. Although this disease was concluded as a kind of precursor disease of gastric cancer at Sydney Conference, the mechanism of carcinogenesis from varioliform gastritis was unknown. Gastric cancer might be effectively controlled if this premalignant lesion-varioliform gastritis-is detected and treated before invasion occurs. Therefore, it is crucial to elucidate the molecular mechanism underlying varioliform gastritis. Some current mechanistic models focus almost on the localized lesion or *H. pylori* infection, with much less attention paid to pathologic changes occurring in the normal-appearing mucosa without *H. pylori* infection from which such lesions emerge.

The pattern of expressed proteins can reflect the information about the functional status and health of the tissue. Recently, the development of new methods for protein analysis has led to the emergence of a new field of clinical proteomics, in which these techniques are harnessed to identify functional molecular or biomarkers of cancer and other diseases[7], but there is hardly any study on the differential expressions of proteins in varioliform gastritis and normal-appearing mucosa.

In the present study, we used proteomic techniques to test the hypothesis that normal gastric mucosa from a patient with varioliform gastritis would exhibit different pattens of protein expression with the disordered mucosa from the same patient. By this approach, comparison of anatomically normal and disordered tissues against the same genetic background could be made.

MATERIALS AND METHODS

Sample collection

Samples were taken from 17 patients with varioliform gastritis in the Second Affiliated Hospital of General Hospital of PLA (Table 1). These patients were examined by ^{13}C urea breath test and the results were all negative. The results of autoantibody detection were also negative in these patients. The case of *H. pylori* infection and autoimmune disease was excluded. Normal gastric mucosa was defined as that 5cm adjacent to the elevations. All samples were obtained by biopsy in endoscopic examinations for these patients. Four pieces of elevatory tissues and normal mucosa were collected from each patient, respectively. One piece of the elevatory tissue underwent pathological diagnosis, and the others were saved for future studies. The patients were well informed in accordance with the disciplines of the Ethics Committee of Biomedicine, General Hospital of PLA, China.

All samples were snap-frozen in liquid nitrogen and stored in a deep freezer (-80℃) until used. Tissues (80-150 mg) were crushed in liquid nitrogen and lysed in 1 mL of 7 mol/L urea, 2 mol/L thiourea, 4% 3-[(3-cholamidopropyl) dimethylammonio] propanesulfonate (CHAPS), 65 mmol/L dithiothreitol (DTT), and 0.2% Bio-Lyte (pH 5-8, Bio-Rad, Hercules, CA) with sonication on ice. The lysates were centrifuged at 20 000 × *g* for 1 h at 4℃. Supernatants were removed and concentrations were determined by the Bio-Rad AC DC protein assay kit (Bio-Rad). The protein samples were stored at -80℃. Before 2-DE was performed, the protein samples were purified using the Readyprep 2-D cleanup kit (Bio-Rad) according to the manufacturer's instructions.

Clinical data of samples

Detailed clinical and pathological data from the health care information center were reviewed. None of the patients had received treatment prior to endoscopic examination. Of the 17 patients, 11 were men, and six were women; the mean age was 51 years (range, 34-72 years, Table 1). No patient suffered from varioliform gastritis with other concurrent gastric diseases. All tissues of varioliform gastritis had definite histologic diagnoses: acute and chronic mucosal inflammation (n = 12), acute and chronic mucosal inflammation with lymphocytic infiltration (n = 5). None of them had *H. pylori* infection or low-to-moderate dysplasia.

Table 1 Characteristics of varioliform gastritis patients in this study

Patient No.	Sex	Age (yr)	Lesion site	Histology
1	F	77	Gastric antrum	Acute and chronic mucosal inflammation, *H. pylori* (-)
2	F	54	Gastric antrum	Acute and chronic mucosal inflammation, *H. pylori* (-)
3	F	59	Gastric body and gastric antrum	Acute and chronic mucosal inflammation with Lymphocytic infiltration, *H. pylori* (-)
4	F	43	Gastric body and gastric antrum	Acute and chronic mucosal inflammation, *H. pylori* (-)
5	F	62	Gastric antrum	Acute and chronic mucosal inflammation, *H. pylori* (-)
6	F	68	Gastric body and gastric antrum	Acute and chronic mucosal inflammation, *H. pylori* (-)
7	M	44	Gastric antrum	Acute and chronic mucosal inflammation with Lymphocytic infiltration, *H. pylori* (-)
8	M	36	Gastric antrum	Acute and chronic mucosal inflammation, *H. pylori* (-)
9	M	76	Gastric antrum	Acute and chronic mucosal inflammation with Lymphocytic infiltration, *H. pylori* (-)
10	M	67	Gastric antrum	Acute and chronic mucosal inflammation, *H. pylori* (-)
11	M	55	Gastric antrum and pylorus	Acute and chronic mucosal inflammation, *H. pylori* (-)
12	M	45	Gastric antrum	Acute and chronic mucosal inflammation, *H. pylori* (-)
13	M	72	Gastric antrum	Acute and chronic mucosal inflammation with Lymphocytic infiltration, *H. pylori* (-)
14	M	57	Gastric antrum and pylorus	Acute and chronic mucosal inflammation, *H. pylori* (-)
15	M	61	Gastric antrum	Acute and chronic mucosal inflammation with Lymphocytic infiltration, *H. pylori* (-)
16	M	51	Gastric antrum	Acute and chronic mucosal inflammation, *H. pylori* (-)
17	M	78	Gastric antrum and pylorus	Acute and chronic mucosal inflammation, *H. pylori* (-)

H. pylori: *Helicobacter pylori*.

Two-dimensional gel electrophoresis

Individual paired samples of normal gastric mucosa and varioliform gastritis were analyzed by 2-DE as described by Xing previously[8]. Briefly, linear gradient 24-cm (pH 5-8) ready strip (Bio-Rad) was rehydrated overnight at 16℃ with 200 μg of protein samples in 500 μL of rehydration buffer (7 mol/L urea, 2 mol/L thiourea, 4% CHAPS, 65 mmol/L DTT, and 0.2% Bio-Lyte). Isoelectric focusing (IEF) was performed using PROTEAN IEF Cell (Bio-Rad). After IEF, the immobilized pH gradient strip was immediately equilibrated in equilibration buffer Ⅰ [6 mol/L urea, 2% sodium dodecyl sulfate (SDS)], 0.375 mol/L Tris-HCl pH 8.8, 20% glycerol, and 2% DTT) for 15 min and then in equilibration buffer Ⅱ (6 mol/L urea, 2% SDS, 0.375 mol/L Tris-HCl pH 8.8, 20% glycerol, and 2.5% iodoacetamide) for 15 min. SDS-polyacrylamide gel electrophoresis was carried out on 12% SDS-polyacrylamide gels (25 cm × 20.5 cm × 1.0 mm) using the PROTEAN Plus Dodeca Cell (Bio-Rad) at a constant voltage of 200 V at 20℃. After electrophoresis, the gels were stained using the Silver Stain Plus Kit (Bio-Rad). The above processes were performed in triplicate for each sample.

Gel imaging and analysis

The silver-stained 2-DE gels were scanned on a GS-800 Calibrated Imaging Densitometer (Bio-Rad) at a resolution of 300 dots per inch. Intensities of protein spots were analyzed with Amersham Biosciences-Imagemaster v5.0. The differential protein spots were defined as those having a 5-fold higher or lower level of differential expression in at least 9 cases compared with the normal mucosa.

Spot cutting and in-gel digestion

The 17 samples were used for spot cutting. Equal protein masses of each sample (normal gastric mucosa and varioliform gastritis tissue) were pooled, and 300 μg of the mixture was loaded for 2-DE. The differentially expressed protein spots were identified as described in the preceding text. These spots were excised from gels by Proteomeworks Spot Cutter (Bio-Rad), destained for 20 min in 30 mmol/L potassium ferricyanide/100 mmol/L sodium thiosulfate [1:1 (v/v)], and washed in Milli-Q water until the gels shrank and bleached. The gel pieces were incubated in 0.2 mol/L NH_4HCO_3 for 20 min and dried by lyophilization. Twenty microliters (20 μg/mL in concentration) trypsin (proteomics grade, Sigma, St. Louis, MO) was added into each gel piece, and incubated at 37℃ overnight. The peptides were extracted three times with 50% acetonitrile and 0.1% trifluoroacetic acid and dried in a vacuum centrifuge.

Mass spectrometry

The digests were analyzed using a Bruker Autoflex Ⅱ TOF/TOF mass spectrometer with delayed extraction in which α-cyano-4-hydroxycinnamic acid was exploited as the matrix. The total 2-μL solution was applied onto a target disk and allowed to air-dry. Mass-to-charge ratios were measured in a reflector/delayed extraction mode with an accelerating voltage of 20 kV, a grid voltage of 63%-65%, positive polarity, and a delay time of 200 nanoseconds. Laser shots at 300 per spectrum were used to acquire the spectra from 800 to 4000 Daltons. Trypsin autolysis products were used for internal mass calibration. Database searching was performed using Mascot software (http://www.matrixscience.com). The search parameters were the nrNCBI database, human, 10-150 kDa, trypsin (1 missed enzymatic cleavage), and 100-ppm mass tolerance. The best match was the one with the highest score, and a significant match was typically a score of more than 70 ($P < 0.05$).

Western blotting analysis

After the analysis of selected proteins, two differential proteins were confirmed by Western blotting analysis in additional samples for validating the 2-DE results. West-

ern blotting analysis was performed in 12 cases of varioliform gastritis with individual-matched normal mucosa. Tissue samples were lysed as described above and protein extracts (50 μg) were separated on a 12% SDS-polyacrylamide gel. Proteins were transferred to a poly-vinylidene difluoride membrane (Bio-Rad). After blocking, the membranes were incubated with a rabbit monoclonal antibody of phosphatidylethanolamine-binding protein 1 (PEBP1) (dilution of 1:2000; Epitomics, California, MA) and polyclonal goat anti-thioredoxin domain-containing protein 5 (TXNDC5) antibody (dilution of 1:1000; Cell Signaling Technology, Danvers, MA). Subsequently, the membranes were incubated in horseradish peroxidase-anti-rabbit and horseradish peroxidase-anti-goat IgG (Abcam, Cambridge, UK), respectively. The specific proteins were visualized with chemiluminescent reagent (Pierce Biotechnology, Rockford, IL). As a control for equal protein loading, blots were restained with anti-actin antibody (dilution of 1:4000; Santa Cruz Biotechnology, Santa Cruz, CA). The band intensity was analyzed by PDQuest software v7.1. The relative expression level was calculated as the intensity ratio of PEBP1 or TXNDC5 to that of actin. The association between categorical data was analyzed using the SPSS11.0 software package.

Reverse transcription polymerase chain reaction of TXNDC5 and PEBP1

The total RNAs of additional samples were extracted by homogenization in Trizol (Invitrogen) for validating the 2-DE results. cDNA synthesis was performed in 20 μL reaction system of reverse transcription including 5 μg RNA. Amplification of TXNDC5, PEBP1 and β2-MG acting as internal control was carried out in DNA thermal cycler (Perkin Elmer) using equal cDNA as template. PCR products were separated by 1.5% agarose gel electrophoresis, scanned and analyzed with VDS ImageMaster system (Pharmacia).

Preliminary functional analysis of TXNDC5 and PEBP1

To understand the function of TXNDC5 and PEBP1 in varioliform gastritis, they were imported into Pathway Studio (demo), and a visualized interaction map was generated with information from Ensembl database, the Pfam protein family database, Prosite database, GNF GeneAtlas database and PDB database. Each node represents either a protein entity or a control mechanism of the interaction. We intended to find the key pathway including TXNDC5, PEBP1 and other proteins in our proteomics research by analyzing the protein interaction networks.

Statistical analysis

SPSS11.0 statistical software was used for the statistical analysis.

The gray values of the protein candidates were analyzed by the nonparametric Wilcoxon test. The intensity ratio of PEBP1 or TXNDC5 to that of internal control in Western blotting or reverse transcription polymerase chain reaction (RT-PCR) analysis was analyzed by one-factor analysis of variance.

RESULTS

Differential protein expression of varioliform gastritis

The 2-DE protein patterns were studied in 17 patients with varioliform gastritis and individual-matched normal mucosa tissues. About 1800 proteins were detected in each gel. The proteins expressed in varioliform gastritis were compared with those in matched normal tissues. The differentially expressed candidates were the protein spots having a 5-fold higher or lower level of differential expression in at least 9 cases (Figure 1A and B). In this study, 21 significantly different candidate protein spots were found. They were also present in the 2-DE gel. Eleven proteins were upregulated and 10 downregulated in varioliform gastritis compared with the same proteins in individual-matched normal gastric mucosa. The quantities of all detected spots were analyzed by the nonparametric Wilcoxon test. These candidate spots were then analyzed by mass spectrometry (MS), and a total of 18 proteins (Figure 1C and Table 2) were identified. We failed to detect three protein spots. There might be several reasons, such as lower abundance, errors in the operation, lower reliability of the MS results, and characteristics of these proteins. More work will be done on the three protein spots in the future studies.

Validation of PEBP1 and TXNDC5 by Western blotting

The two novel candidate proteins, PEBP1 and TXNDC5, were studied further among the differentially expressed proteins. Their expression profiles in varioliform gastritis have not been reported previously. Western blotting analysis showed that TXNDC5 was upregulated significantly in varioliform gastritis but not in normal gastric mucosa (mean ± SD: 0.37 ± 0.05) (Figure 2B). Compared with that in normal mucosa (mean ± SD: 0.76 ± 0.12), PEBP1 was significantly downregulated in varioliform gastritis (mean ± SD: 0.18 ± 0.08) ($P < 0.05$, by Student's test or the Friedman test) (Figure 2A).

Validation of PEBP1 and TXNDC5 by RT-PCR

Using semiquantitative RT-PCR, 476 bp fragment of TXNDC5, 451 bp fragment of PEBP1 and 876 bp control fragment of β2-MG were amplified (Figure 3). The mean ratios of the absorbency of PEBP1 band normalized to the control band were 0.35 ± 0.09 and 1.23 ± 0.27 in 12 cases of varioliform gastritis and normal mucosa. *P* value was lower than 0.05 when Student's *t* test was used to compare the ratios of the two groups (Figure 3B). Those of TXNDC5 were 1.15 ± 0.07 and 0.23 ± 0.06 in 12 cases of varioliform gastritis and normal mucosa, respectively ($P < 0.05$) (Figure 3A). The results suggested that the difference of TXNDC5 and PEBP1 between varioliform gastritis and normal mucosa could be obvious at the mRNA level.

Preliminary functional analysis

TXNDC5 and PEBP1 were imported into Pathway Studio (demo) to build an interaction network. The connectivity of TXNDC5 and PEBP1 was 40 and 145, respectively. The average connectivity of proteins identified was about

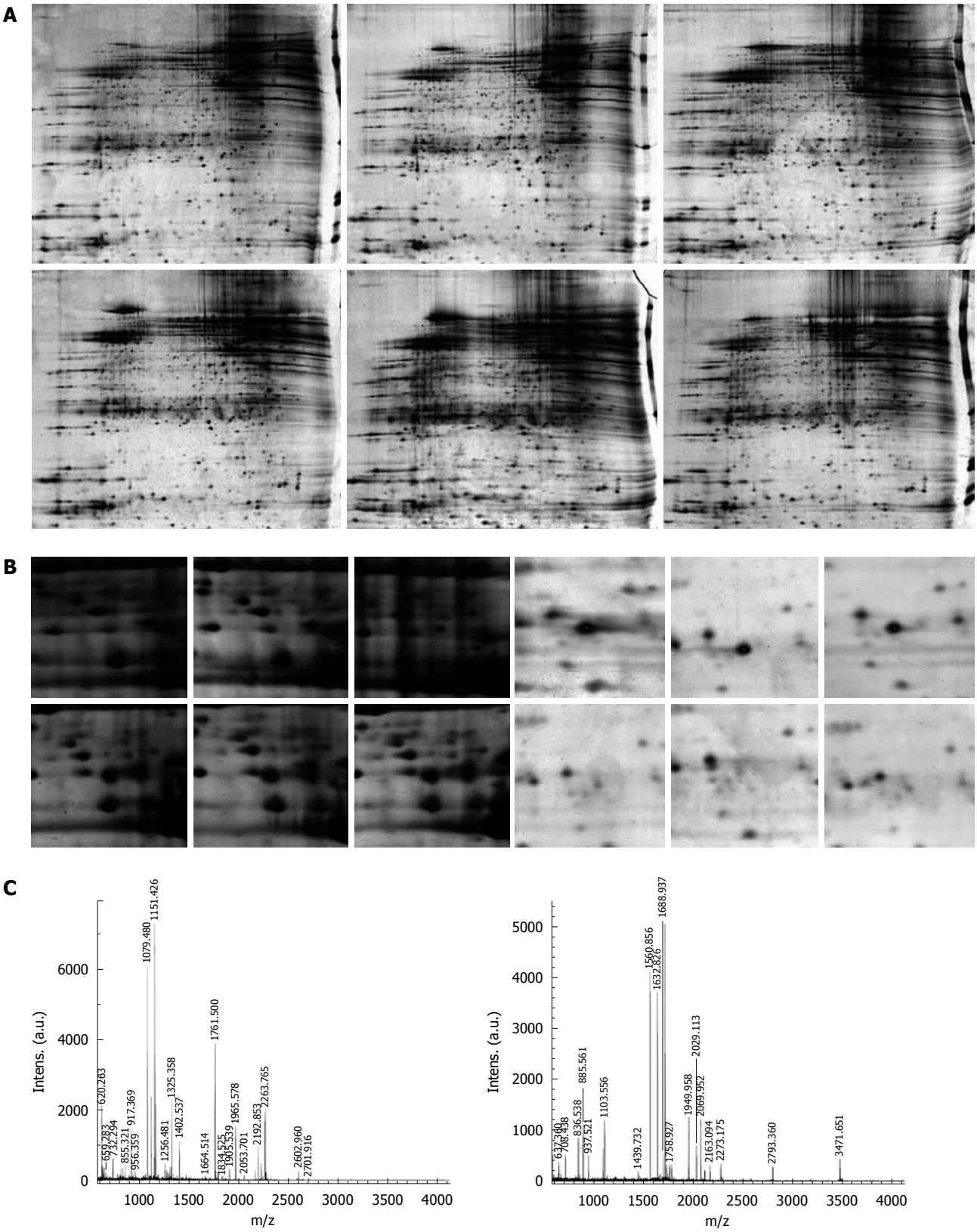

Figure 1 Detection and analysis of differentially expressed proteins in varioliform gastritis. A: Representative 2-DE images of matched varioliform gastritis and normal gastric mucosa tissue. The proteins expressed in varioliform gastritis were compared with those expressed in matched normal tissue. The protein spots that showed more than 5-fold differential expression in at least nine cases were taken as differentially expressed candidates. Of 21 differentially expressed protein spots, 18 were identified by mass spectrometry (MS) (protein nomenclature can be seen in Table 2); B: The magnified regions of the 2-DE gel of upregulated thioredoxin domain-containing protein 5 (TXNDC5) (left) and downregulated phosphatidylethanolamine-binding protein 1 (PEBP1) (right) in varioliform gastritis, compared with normal tissue; C: MS of in-gel trypsin digests of these proteins and analysis of the depicted peptide spectrum resulted in the identification of TXNDC5 (left) and PEBP1 (right).

Table 2 Differentially expressed proteins in varioliform gastritis

ID No.	Protein name	Gene name	Accession No.	Mass (Da)/pI	Cover rate (mean, %)	Mascot scores	Intensity of candidate protein spots[a] (positive rate)	General function/comments
	Up-regulated proteins							
1	Thioredoxin domain containing protein 5 precursor	*TXNDC5*	Q8NBS9	47629.2/5.3	25	228	36.5 ± 3.1, 2.7 ± 0.4 (100%, 44.4%)	Controlling the oxidative protein folding in endoplasmic reticulum
2	Proliferating cell nuclear antigen	*PCNA*	P12004	28762.4/4.7	37	184	18.6 ± 4.2, 3.3 ± 0.9 (94.4%, 70.5%)	Cell growth and maintenance
3	40S ribosomalprotein SA	*RPSA*	P08865	32702.4/4.79	46	104	64.3 ± 11.1, 11.5 ± 2.8 (100%, 88.3%)	Originally known as laminin receptor precursor and p40
4	Heat-shock protein β-1	*HSPB1*	P04792	22768.5/5.9	33		73.8 ± 13.4, 12.7 ± 3.4 (100%, 100%)	A HSP27 isoform (pI5.68)
5	Inorganic pyrophosphatase2	*PPA2*	Q9H2U2	35472/5.9	69	245	24.1 ± 2.7, 3.6 ± 1.2 (94.4%, 58.9%)	Overexpressed in some cancer tissues
6	S100 calcium-binding protein A10	*S100A10*	P60903	11195.5/6.8	62	148	43.0 ± 5.7, 5.9 ± 2.7 (88.9%, 41.2%)	It may function as a regulator of protein phosphorylation in the ANXA2 monomer
7	Nucleoside diphosphate kinase A	*NME1*	P15531	17137.7/5.8	49	193	59.4 ± 12.6, 13.2 ± 5.8 (82.4%, 70.6%)	It plays a major role in the synthesis of nucleoside triphosphates other than ATP
8	Proteasome activator complex subunit 1	*PSME1*	Q06323	38966.2/7.6	53	138	83.8 ± 17.4, 10.5 ± 4.7 (100%, 41.2%)	Implicated in immuno-proteasome assembly and required for efficient antigen processing
9	Ubiquitin thiolesterase L3	*UCHL3*	P15374	26337/4.7	42	174	62.4 ± 11.9, 13.7 ± 7.1 (88.9%, 29.4%)	Ubiquitin-protein hydrolase involved in the processing of both ubiquitin precursors and ubiquitinated proteins
10	S100 calcium-binding protein A6	*S100A6*	P06703	11732.8/5.6	71	234	21.6 ± 5.3, 3.7 ± 1.1 (82.4%, 23.5%)	Preferentially expressed when quiescent fibroblasts are stimulated to proliferate
	Down-regulated proteins							
11	Cell division cycle 2-like protein kinase 5	*CDC2L5*	Q14004	48212.2/8.3	37	168	1.7 ± 0.5, 14.2 ± 2.6 (17.6%, 52.9%)	May be a controller of the mitotic cell cycle involved
12	BTG3 protein	*BTG3*	Q14201	29117.3/9.1	64	265	5.8 ± 2.3, 47.4 ± 6.1 (29.4%, 82.4%)	Overexpression impairs serum-induced cell cycle progression from the G0/G1 to S phase
13	Neuropolypeptide h3	*PEBP1*	P30086	31270.6/5.7	58	220	2.9 ± 1.4, 58.6 ± 11.8 (17.6%, 100%)	Binds ATP, opioids and phosphatidylethanolamine
14	Heat-shock protein 17 kDa	*HSPB3*	Q12988	16966/5.7	39	105	16.8 ± 4.4, 89.5 ± 14.7 (100%, 100%)	Inhibitor of actin polymerization
15	Caspase-5 precursor	*CASP5*	P51878	47815/9.2	48	201	7.6 ± 2.6, 45.9 ± 11.7 (41.2%, 77.8%)	Mediator of apoptosis
16	Cytokeratin 20	*KRT20*	P35900	48487/4.9	52	131	2.9 ± 0.4, 17.5 ± 3.6 (29.4%, 52.9%)	It plays a significant role in maintaining keratin filament organization in intestinal epithelia. When phosphorylated, it plays a role in the secretion of mucin in the small intestine
17	Eukaryotic translation initiation factor 3 subunit 2	*EIF3I*	Q13347	23354/4.9	44	173	8.9 ± 2.3, 46.3 ± 17.2 (35.3%, 100%)	Binds to the 40S ribosome and promotes the binding of methonyl
18	Ribosomal protein S12	*RPS12*	P25398	14526.0/5.6	56	141	5.7 ± 1.4, 52.6 ± 10.3 (29.4%, 70.6%)	Belongs to the ribosomal protein S12e family

[a]$P < 0.05$ *vs* the normal gastric mucosa. *t* test was used for analyzing the difference of the intensity of candidate protein spots. PEBP1: Phosphatidylethanolamine-binding protein 1; TXNDC5: Thioredoxin domain-containing protein 5.

57. Our results showed that some members of mitogen-activated protein kinase (MAPK) family and some molecules involved in nuclear factor (NF)-κB and tumor necrosis factor were hot points with higher connectivity. From the mimical molecular network, we concluded that NF-κB, MAPK and interferon γ (IFN-γ) pathways were the cores of the whole network. The downstream-related cancer and other phenotypes were linked to the three pathways. The reaction of cell to IFN-γ could be the initiating agent of this molecular interaction network (Figure 4).

DISCUSSION

Because of limited knowledge on varioliform gastritis, the molecular events underlying this disease were still unknown, and the patients could be faced with more risk of gastric cancer. It was confirmed that the mucosal lesion of varioliform gastritis could develop into malignant tumor. Proteomic studies can help understand the early stages in the genesis of varioliform gastritis and has the potential to aid in the prevention and intervention for gastric can-

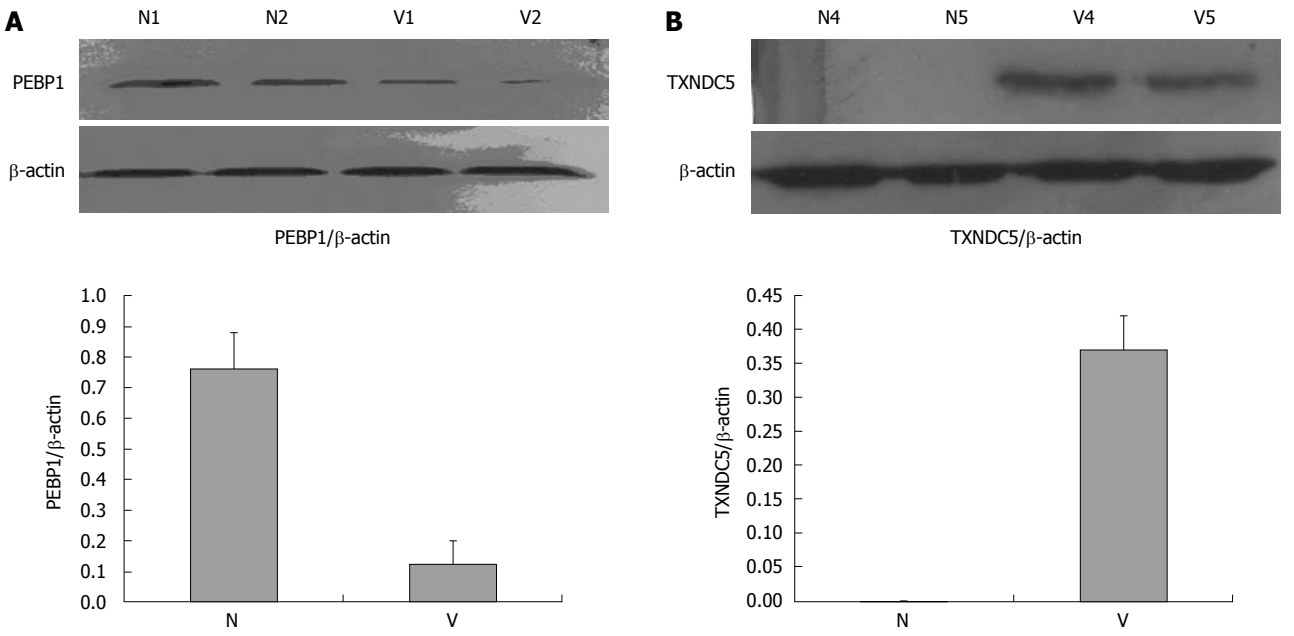

Figure 2 Western blotting analysis of phosphatidylethanolamine-binding protein 1 and thioredoxin domain-containing protein 5. A: Marked downregulation of phosphatidylethanolamine-binding protein 1 (PEBP1) in varioliform gastritis (V) tissue. Protein extracts (50 μg) were separated on a 12% sodium dodecyl sulfate-polyacrylamide gel. Proteins were transferred to a poly-vinylidene difluoride membrane. After blocking, the membranes were incubated with rabbit monoclonal antibody of PEBP1 (dilution of 1:2000) and subsequently incubated with HRP-anti-rabbit IgG. The specific proteins were visualized with chemiluminescent reagent. As a control for equal protein loading, blots were restained with anti-actin antibody. Immunosignals were quantified by densitometry scanning. The relative quantification was calculated as the ratio of PEBP1 expression to actin expression as shown in the followed chart; B: Upregulation of thioredoxin domain-containing protein 5 (TXNDC5) in varioliform gastritis in comparison with that in normal (N) mucosa. The same experimental process was performed, except that the membranes were incubated with polyclonal goat anti-TXNDC5 antibody (dilution of 1:1000).

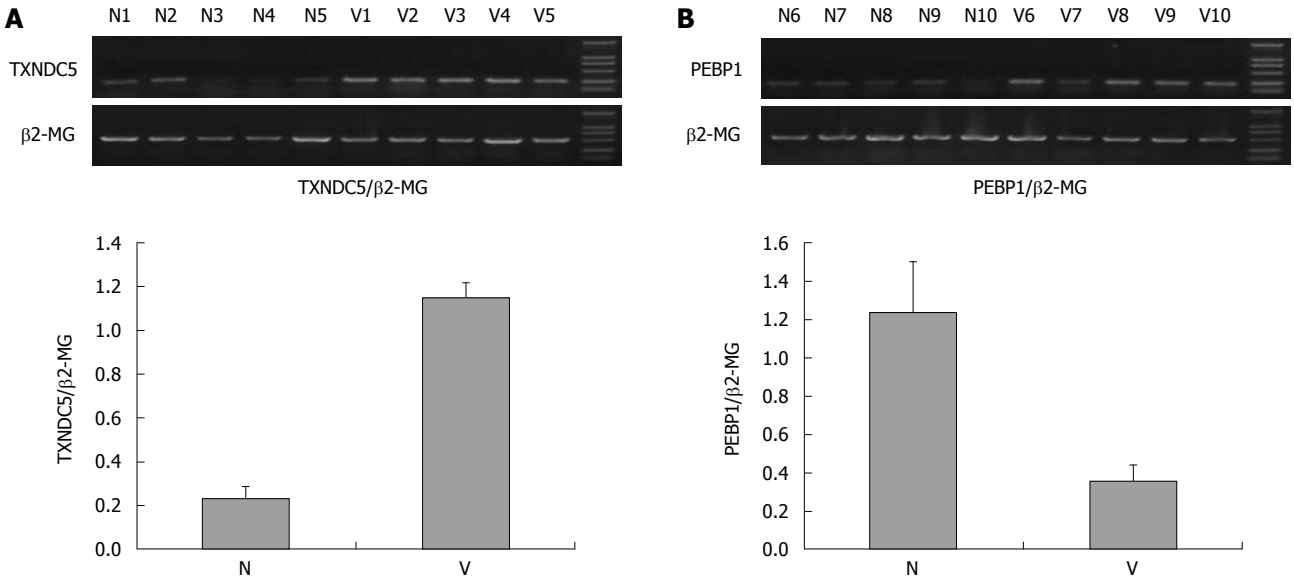

Figure 3 Reverse transcription polymerase chain reaction analysis of thioredoxin domain-containing protein 5 and phosphatidylethanolamine-binding protein 1. A: Marked upregulation of thioredoxin domain-containing protein 5 (TXNDC5) in varioliform gastritis (V). The total RNAs of additional samples tissues were extracted by homogenization in Trizol (Invitrogen), cDNA synthesis was performed in 20 μL reaction system of reverse transcription including 5 μg RNA. Amplification of TXNDC5, with β2-MG acting as internal control, was carried out in DNA thermal cycler. PCR products were separated by 1.5% agarose gel electrophoresis. The bands were quantified by densitometry scanning. The relative quantification was calculated as the ratio of TXNDC5 expression to β2-MG expression as shown in the followed chart; B: Downregulation of phosphatidylethanolamine-binding protein 1 (PEBP1) in varioliform gastritis in comparison with that in normal (N) mucosa. The same experimental process was performed. The relative quantification was calculated as the ratio of PEBP1 expression to β2-MG expression as shown in the followed chart.

cer. In this study, we used the common approach of 2-DE coupled with MS to study the differentially expressed proteins in individual-matched cases of normal mucosa and lesion of varioliform gastritis and confirmed the differential expression of PEBP1 and TXNDC5 by Western blotting or RT-PCR.

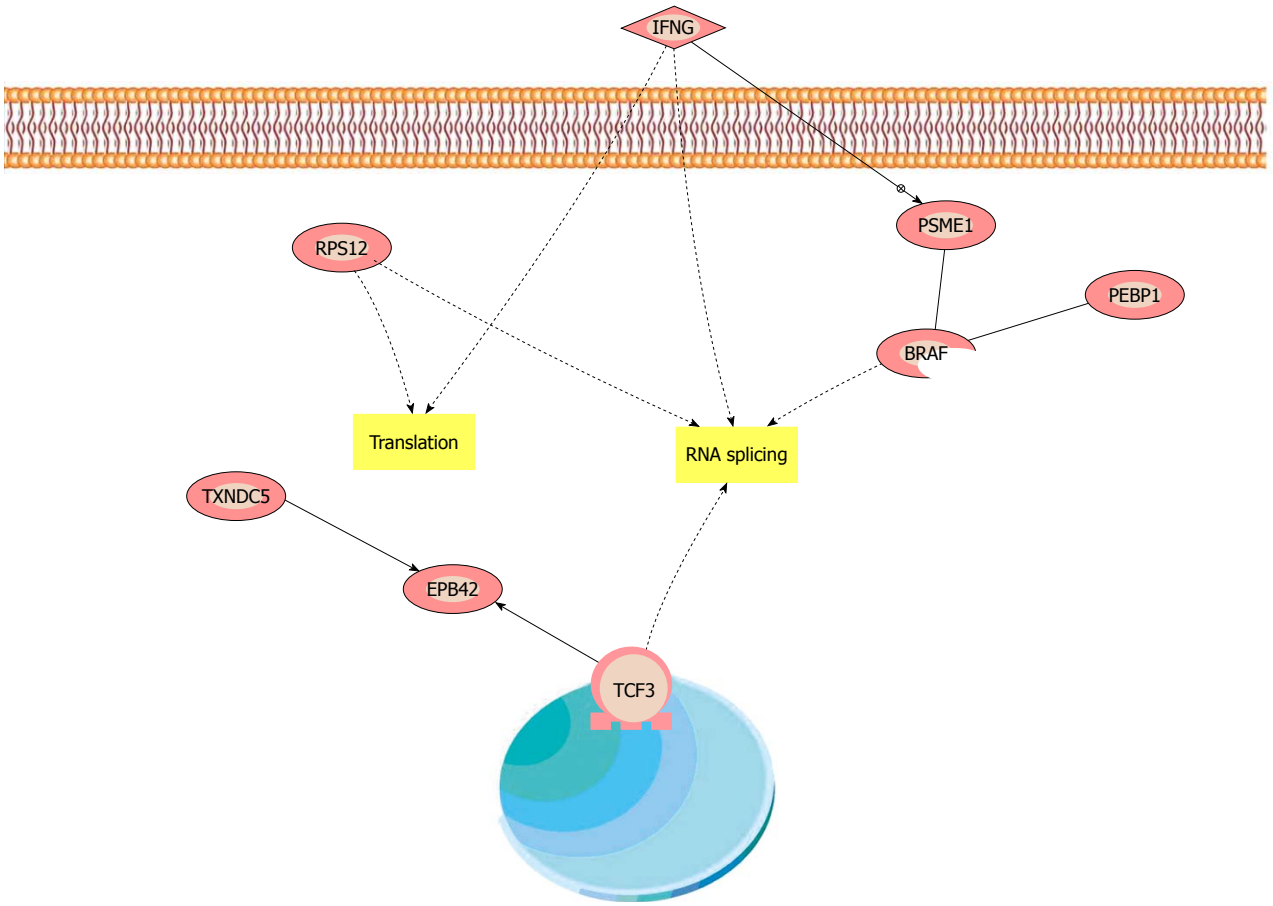

Figure 4 Compendious biological interaction networking of phosphatidylethanolamine-binding protein 1 and thioredoxin domain-containing protein 5 in varioliform gastritis. Phosphatidylethanolamine-binding protein 1 (PEBP1) and thioredoxin domain-containing protein 5 (TXNDC5) were imported into Pathway Studio (demo), and an interaction map was generated. Compendious molecular interaction pathway which linked PEBP1, TXNDC5 and interaction pathways and interferon γ (INFG). INFG could induce some complex molecular interaction in cells and impact on cell proliferation and apoptosis, and then could promote the formation of varioliform lesion.

A concept in cancer biology is that tumors arise and grow from some precancerous lesions as a result of the multiple changes of the genes or proteins which could influence the functions of cells *via* different molecular biological pathways. So it could be very important to find out these molecular changes and their functional pathways. The changes can be detected and analyzed in genomics and proteomics. A differential protein expression profile is a snapshot of the proteomics composition of a specific tissue at a specific time, which can be a key clue for further studies on the underlying mechanisms.

In this study, we identified 21 differentially expressed proteins in varioliform gastritis. However, none of these proteins (Table 2) had been reported in previous studies on this disease. We used a 5-fold cut-off according to the previous studies[9,10], and only found 21 differentially expressed proteins between varioliform gastritis and normal mucosa in the 2-DIGE study. We believe that some major molecular mechanisms underlying the disease should be implicated. There are also some methodological discrepancies in the process of our proteomic study, including the sample collection, the separation and identification of proteins and the analysis of results. Some low-abundance protein spots could not be displayed clearly, which should be further analyzed by a more advanced method.

PEBP1 expression was strong in normal mucosa, but significantly downregulated in varioliform gastritis. An alternative name of PEBP1 was Raf kinase inhibitory protein (RKIP) that belongs to the PEBP family. It is an inhibitor of the Raf/MEK/MAP kinase signaling cascade and is a suppressor of cancer metastasis[11]. Some researches[12,13] have confirmed that PEBP1 regulates activation of MAPK, NF-κB and G protein coupled receptors. As a modulator of key signaling pathways, PEBP1 affects various cellular processes, including cell differentiation, the cell cycle, apoptosis and cell migration. To date, emerging evidence[14-21] suggests that PEBP1 plays a crucial suppressing role in tumorigenesis and metastasis of prostate cancer, ovarian cancer, cervical cancer, colorectal cancer, liver cancer and breast cancer. It represents a novel effector of signal transduction pathways leading to apoptosis and a prognostic marker of the pathogenesis of human cancer cells and tumors. Chatterjee *et al*[22] have examined the expression patterns of PEBP1 and STAT3 in samples from 143 patients with gastric adenocarcinoma using tissue microarrays. Their results indicate the predictive and protective role of PEBP1 expression in gastric adenocarcinoma of the intestinal subtype. Downregulated expressions of PEBP1 could decrease patients' survival. Collectively, these studies suggest that the PEBP1 or RKIP gene, as a poten-

tial tumor suppressor gene, is involved in gastric cancer initiation and progression, and expression of PEBP1 could be downregulated at initiation of tumorigenesis. Our results confirmed that PEBP1 expression was downregulated in varioliform gastritis compared with that of normal mucosa. We, therefore, postulate that this downregulation of PEBP1 might denote a step of the potential canceration.

TXNDC5 was significantly upregulated in varioliform gastritis although its expression remained in normal mucosa. TXNDC5 was first detected by 2-DE analysis of the luminal environment of the endoplasmic reticula of hepatic tissues in 2003[23]. As a novel PDI-like protein, TXNDC5 was highly expressed in endothelial cells. This tissue-specific expression is unusual among members of the PDI family. Sullivan *et al*[24] have confirmed that TXNDC5 could protect endothelial cells from stress-induced apoptosis. In contrast to PDI, which is essential for the survival of endothelial cells in the resting as well as the stressed state, TXNDC5 protects endothelial cells only under conditions of stress. They have found that loss of TXNDC5 results in reduced secretion of adrenomedullin and endothelin-1 together with a reduction in membrane-bound CD105, while TXNDC5 is essential for folding of CD105. The results of Edman's and Freedman's researches[25,26] suggested that TXNDC5 could play important roles in antioxidative injury, antianoxia-induced apoptosis, and promotion of proliferation in cells. Some recent studies showed that upregulation of TXNDC5 was found in tumors of the cervix, uterus, stomach and lung[24]. Nissom *et al*[27] found that a variant of the TXNDC5 gene was upregulated in poorly differentiated hepatocellular carcinoma (HCC) but unchanged in well-differentiated HCC. According to these reports, we think that TXNDC5 gene could be a tumor-enhancing gene, but the detailed biological roles of TXNDC5 in varioliform gastritis and gastric cancer remain to be elucidated. The upregulation of TXNDC5 in varioliform gastritis suggests that this disease could be related to gastric cancer, with higher risk than what was thought before.

The NF-κB and MAPK signaling pathways regulate growth in many tumors or inflammation, suggesting the cooperative role of these two pathways in the regulation of cell proliferation and apoptosis. *H. pylori* is known to be the cause of most gastric diseases, including both peptic ulcer disease and gastric cancer. Fox *et al*[28] think that the induction by *H. pylori* of cytokines and chemokines and growth-related genes is mediated by the MAPK and NF-κB signaling pathways, and Shibata *et al*[29] and Lee *et al*[30] have confirmed this conclusion. Kacar *et al*[31] and Chen *et al*[32] found that MAPK signaling pathway could be a causative factor in the alterations of the gastric mucosa infected by *H. pylori* and MAPK activation seems to be a significant and persistent event in the *H. pylori*-induced neoplastic transformation. IFN-γ acts through distinct cell surface receptors and induces transcription of an overlapping sets of genes. MHC class I genes are inducible by this interferon. IFN-γ is the gastric mucosal immunological reaction produced by T helper cells when gastric mucosa is infected by *H. pylori*[33-35]. It could induce the changes of TXNDC5 or PEBP1 and impact on the NF-κB and MAPK signaling pathways in our molecular interaction network of varioliform gastritis. We supposed that varioliform gastritis should be the results of a series of molecular interactions induced by IFN-γ or other molecules as an immunological reaction against microorganism infection according to previous reports and our research. But most of the previous studies focused on *H. pylori*, and the researches on other pathogens were very limited. So there could be some other bacteria or viruses which could induce an analogous immunological reaction against *H. pylori* in the gastric mucosa of varioliform gastritis patients without *H. pylori* infection.

In summary, our study showed a differential protein expression profile of varioliform gastritis compared with that of matched normal mucosa. The candidate proteins may confirm the previous conclusion that varioliform gastritis is one of the major precursor diseases of gastric cancer. The risk of potential canceration could be higher than what was thought previously, so effective treatment strategies should be studied and adopted for this disease in the future.

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COMMENTS

Background

Varioliform gastritis is a chronic gastritis with a potential of developing into gastric cancer. To date, the etiopathogeny of the disease is unclear. So it is crucial to elucidate the molecular mechanism underlying the disease for preventing gastric cancer.

Research frontiers

At present, the researches on varioliform gastritis focused mostly on endoscopic diagnosis and treatment or clinical feature. The reports on the molecular mechanism of the disease are very limited. As a new field of clinical proteomics is emerging, many new techniques have been developed to identify functional molecules or biomarkers of cancer and other diseases.

Innovations and breakthroughs

In this research, a differential protein expression profile of varioliform gastritis was indicated compared with that of matched normal mucosa. The important differential proteins and potential signal pathways have been provided for the future studies.

Applications

The results of this study provided some valuable clues for elucidating the molecular mechanism of varioliform gastritis and the relationship between the disease and gastric cancer. Some potential biomarkers were indicated for the early diagnosis of gastric cancer and therapeutic targets for this tumor.

Terminology

Varioliform gastritis: a special kind of chronic gastritis characterized by nodules, thickened fugal folds and erosions. Although it is a very common gastritis, its features appear to be unusual and different from those seen in chronic gastritis.

Peer review

The authors used proteomic techniques to identify differences in protein expression patterns in normal gastric mucosa *vs* mucosa characterized by varioliform gastritis. The study is well designed, represents a large amount of work, and will potentially be very helpful to further studies in the field of cancer research and treatment.

REFERENCES

1 **Parkin DM**, Pisani P, Ferlay J. Global cancer statistics. *CA Cancer J Clin* 1999; **49**: 33-64, 1

2 **Munoz Monteavaro C**, Mendoza D, Palma E. [Varioliform gastritis with "in situ" carcinomatous transformation.] *An Fac Med Univ Repub Montev Urug* 1960; **45**: 72-77
3 **Cappell MS**, Green PH, Marboe C. Neoplasia in chronic erosive (varioliform) gastritis. *Dig Dis Sci* 1988; **33**: 1035-1039
4 **Gallina F**, Benedetti-Valentini F. [Varioliform gastritis associated with gastric ulcer simulating a neoplasm.] *Riv Gastroenterol* 1963; **15**: 85-94
5 **Vandenborre KM**, Ghillebert GL, Rutgeerts LJ, Geboes KR, Rutgeerts PJ, Verbanck JJ, Tanghe WR. Hypertrophic lymphocytic gastritis with a gastric carcinoma. *Eur J Gastroenterol Hepatol* 1998; **10**: 797-801
6 **Mosnier JF**, Flejou JF, Amouyal G, Gayet B, Molas G, Henin D, Potet F. Hypertrophic gastropathy with gastric adenocarcinoma: Menetrier's disease and lymphocytic gastritis? *Gut* 1991; **32**: 1565-1567
7 **Wulfkuhle JD**, Liotta LA, Petricoin EF. Proteomic applications for the early detection of cancer. *Nat Rev Cancer* 2003; **3**: 267-275
8 **Xing X**, Lai M, Gartner W, Xu E, Huang Q, Li H, Chen G. Identification of differentially expressed proteins in colorectal cancer by proteomics: down-regulation of secretagogin. *Proteomics* 2006; **6**: 2916-2923
9 **Stulík J**, Hernychová L, Porkertová S, Knízek J, Macela A, Bures J, Jandik P, Langridge JI, Jungblut PR. Proteome study of colorectal carcinogenesis. *Electrophoresis* 2001; **22**: 3019-3025
10 **Roblick UJ**, Hirschberg D, Habermann JK, Palmberg C, Becker S, Krüger S, Gustafsson M, Bruch HP, Franzén B, Ried T, Bergmann T, Auer G, Jörnvall H. Sequential proteome alterations during genesis and progression of colon cancer. *Cell Mol Life Sci* 2004; **61**: 1246-1255
11 **Dangi-Garimella S**, Yun J, Eves EM, Newman M, Erkeland SJ, Hammond SM, Minn AJ, Rosner MR. Raf kinase inhibitory protein suppresses a metastasis signalling cascade involving LIN28 and let-7. *EMBO J* 2009; **28**: 347-358
12 **Odabaei G**, Chatterjee D, Jazirehi AR, Goodglick L, Yeung K, Bonavida B. Raf-1 kinase inhibitor protein: structure, function, regulation of cell signaling, and pivotal role in apoptosis. *Adv Cancer Res* 2004; **91**: 169-200
13 **Zeng L**, Imamoto A, Rosner MR. Raf kinase inhibitory protein (RKIP): a physiological regulator and future therapeutic target. *Expert Opin Ther Targets* 2008; **12**: 1275-1287
14 **Woods Ignatoski KM**, Grewal NK, Markwart SM, Vellaichamy A, Chinnaiyan AM, Yeung K, Ray ME, Keller ET. Loss of Raf kinase inhibitory protein induces radioresistance in prostate cancer. *Int J Radiat Oncol Biol Phys* 2008; **72**: 153-160
15 **Li HZ**, Wang Y, Gao Y, Shao J, Zhao XL, Deng WM, Liu YX, Yang J, Yao Z. Effects of raf kinase inhibitor protein expression on metastasis and progression of human epithelial ovarian cancer. *Mol Cancer Res* 2008; **6**: 917-928
16 **Biewenga P**, Buist MR, Moerland PD, Ver Loren van Themaat E, van Kampen AH, ten Kate FJ, Baas F. Gene expression in early stage cervical cancer. *Gynecol Oncol* 2008; **108**: 520-526
17 **Minoo P**, Zlobec I, Baker K, Tornillo L, Terracciano L, Jass JR, Lugli A. Loss of raf-1 kinase inhibitor protein expression is associated with tumor progression and metastasis in colorectal cancer. *Am J Clin Pathol* 2007; **127**: 820-827
18 **Al-Mulla F**, Hagan S, Behbehani AI, Bitar MS, George SS, Going JJ, García JJ, Scott L, Fyfe N, Murray GI, Kolch W. Raf kinase inhibitor protein expression in a survival analysis of colorectal cancer patients. *J Clin Oncol* 2006; **24**: 5672-5679
19 **Lee HC**, Tian B, Sedivy JM, Wands JR, Kim M. Loss of Raf kinase inhibitor protein promotes cell proliferation and migration of human hepatoma cells. *Gastroenterology* 2006; **131**: 1208-1217
20 **Schuierer MM**, Bataille F, Weiss TS, Hellerbrand C, Bosserhoff AK. Raf kinase inhibitor protein is downregulated in hepatocellular carcinoma. *Oncol Rep* 2006; **16**: 451-456
21 **Hagan S**, Al-Mulla F, Mallon E, Oien K, Ferrier R, Gusterson B, García JJ, Kolch W. Reduction of Raf-1 kinase inhibitor protein expression correlates with breast cancer metastasis. *Clin Cancer Res* 2005; **11**: 7392-7397
22 **Chatterjee D**, Sabo E, Tavares R, Resnick MB. Inverse association between Raf Kinase Inhibitory Protein and signal transducers and activators of transcription 3 expression in gastric adenocarcinoma patients: implications for clinical outcome. *Clin Cancer Res* 2008; **14**: 2994-3001
23 **Clissold PM**, Bicknell R. The thioredoxin-like fold: hidden domains in protein disulfide isomerases and other chaperone proteins. *Bioessays* 2003; **25**: 603-611
24 **Sullivan DC**, Huminiecki L, Moore JW, Boyle JJ, Poulsom R, Creamer D, Barker J, Bicknell R. EndoPDI, a novel protein-disulfide isomerase-like protein that is preferentially expressed in endothelial cells acts as a stress survival factor. *J Biol Chem* 2003; **278**: 47079-47088
25 **Edman JC**, Ellis L, Blacher RW, Roth RA, Rutter WJ. Sequence of protein disulphide isomerase and implications of its relationship to thioredoxin. *Nature* 1985; **317**: 267-270
26 **Freedman RB**, Hirst TR, Tuite MF. Protein disulphide isomerase: building bridges in protein folding. *Trends Biochem Sci* 1994; **19**: 331-336
27 **Nissom PM**, Lo SL, Lo JC, Ong PF, Lim JW, Ou K, Liang RC, Seow TK, Chung MC. Hcc-2, a novel mammalian ER thioredoxin that is differentially expressed in hepatocellular carcinoma. *FEBS Lett* 2006; **580**: 2216-2226
28 **Fox JG**, Wang TC. Helicobacter pylori infection: pathogenesis. *Curr Opin Gastroenterol* 2002; **18**: 15-25
29 **Shibata W**, Hirata Y, Ogura K, Omata M, Maeda S. [NF-kappaB and MAPK-signaling pathways contribute to the gene expression and host response induced by Helicobacter pylori infection] *Nippon Rinsho* 2005; **63** Suppl 11: 132-137
30 **Lee JS**, Kim HS, Hahm KB, Sohn MW, Yoo M, Johnson JA, Surh YJ. Inhibitory effects of 7-carboxymethyloxy-3',4',5-trimethoxyflavone (DA-6034) on Helicobacter pylori-induced NF-kappa B activation and iNOS expression in AGS cells. *Ann N Y Acad Sci* 2007; **1095**: 527-535
31 **Kacar F**, Meteoğlu I, Yasa H, Levi E. Helicobacter pylori-induced changes in the gastric mucosa are associated with mitogen-activated protein kinase (MAPK) activation. *Appl Immunohistochem Mol Morphol* 2007; **15**: 224-228
32 **Chen YC**, Wang Y, Li JY, Xu WR, Zhang YL. H pylori stimulates proliferation of gastric cancer cells through activating mitogen-activated protein kinase cascade. *World J Gastroenterol* 2006; **12**: 5972-5977
33 **Cam S**, Ertem D, Bahceciler N, Akkoc T, Barlan I, Pehlivanoglu E. The interaction between Helicobacter pylori and atopy: does inverse association really exist? *Helicobacter* 2009; **14**: 1-8
34 **Shimada M**, Ando T, Peek RM, Watanabe O, Ishiguro K, Maeda O, Ishikawa D, Hasegawa M, Ina K, Ohmiya N, Niwa Y, Goto H. Helicobacter pylori infection upregulates interleukin-18 production from gastric epithelial cells. *Eur J Gastroenterol Hepatol* 2008; **20**: 1144-1150
35 **Vivas JR**, Regnault B, Michel V, Bussière FI, Avé P, Huerre M, Labigne A, D' Elios MM, Touati E. Interferon gamma-signature transcript profiling and IL-23 upregulation in response to Helicobacter pylori infection. *Int J Immunopathol Pharmacol* 2008; **21**: 515-526

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ORIGINAL ARTICLE

Magnetically labeled mesenchymal stem cells after autologous transplantation into acutely injured liver

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Abstract

AIM: To evaluate tracking of magnetically labeled mesenchymal stem cells (MSCs) after intraportal transplantation.

METHODS: Mononuclear cells were isolated from bone marrow aspirates of pigs by density gradient centrifugation, cultured and expanded, after which, they were incubated with super paramagnetic iron oxide (SPIO). Prussian blue staining was performed to highlight intracellular iron. To establish swine models of acute liver injury, 0.5 g/kg D-galactosamine was administrated to 10 pigs, six of which were injected *via* their portal veins with SPIO-labeled MSCs, while the remaining four were injected with unlabeled cells. Magnetic resonance imaging (MRI) was performed with a clinical 1.5T MR scanner immediately before transplantation and 6 h, 3 d, 7 d and 14 d after transplantation. Prussian blue staining was again performed with the tissue slices at the endpoint.

RESULTS: Prussian blue staining of SPIO-labeled MSCs had a labeling efficiency of almost 100%. Signal intensity loss in the liver by SPIO labeling on the FFE (T2*WI) sequence persisted until 14 d after transplantation. Histological analysis by Prussian blue staining confirmed homing of labeled MSCs in the liver after 14 d; primarily distributed in hepatic sinusoids and liver parenchyma.

CONCLUSION: MSCs were successfully labeled with SPIO *in vitro*. MRI can monitor magnetically labeled MSCs transplanted into the liver.

Key words: Magnetic resonance imaging; Mesenchymal stem cells; Super paramagnetic iron oxide; Stem cell transplantation

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INTRODUCTION

In recent years, cell transplantation has had the advantages of lower cost, lower risk, and simpler manipulation of the procedure compared with orthotopic liver transplantation. A large body of evidence has suggested that mesenchymal stem cells (MSCs) could differentiate into liver-like cells with partial hepatic functions under appropriate environmental conditions *in vivo* and *in vitro*[1,2]. Given that autolo-

gous cell transplantation helps prevent immunological rejection, which is always a problem for orthotopic liver transplantation, MSCs can be regarded as seeding cells for transplantation in relation to liver diseases.

The major issue in liver cell transplantation is monitoring migration, distribution, and differentiation of the transplanted cells. Conventional tissue slicing is unable to distinguish between transplanted donor cells and the recipient cells, therefore, the tissue or organ has to be removed at certain time points and processed with special biochemical procedures to visualize the tagged cells. However, these tagging methods require *in vitro* preparation and examination of histological materials, which are unsuitable for noninvasive and repeated monitoring of *in vivo* transplanted cells under clinical conditions. Therefore, more recent research has focused on *in vivo* real-time tracking and detecting the fate of transplanted cells by using appropriate imaging technologies[3].

The present study had two purposes. First, we incubated swine autologous MSCs with super paramagnetic iron oxide (SPIO) *in vitro*, followed by stem cell transplantation performed *via* the portal vein in acutely injured liver models. Second, we investigated the characteristics of magnetically labeled swine MSCs by magnetic resonance imaging (MRI), as well as intrahepatic dynamic distribution.

MATERIALS AND METHODS

Animal care

Ten outbred white swine of either sex weighing 15-20 kg each were maintained under conventional conditions in the Laboratory Animal Center of the Affiliated Drum Tower Hospital of Nanjing University Medical School. All animal procedures were approved by the Animal Care Ethics Committee of Nanjing Drum Tower Hospital.

MSC isolation, culture and characterization

Porcine MSCs were isolated by bone marrow aspirates from the iliac crests of the animals as previously described, with slight modification[4]. Mononuclear cells were collected by gradient centrifugation over a Ficoll histopaque layer (20 min, 400 *g*, density 1.077 g/mL) (TBD, China) and seeded at a density of 1×10^6 cells/cm^2 in growth medium that contained low-glucose Dulbecco's modified Eagle's medium (DMEM-LG; GIBCO, USA) supplemented with 10% fetal bovine serum (FBS; GIBCO, USA), penicillin (100 IU/mL) and streptomycin (100 μg/mL). The non-adherent cells were removed after the first 24 h and changed every 3-4 d thereafter. When the cells reached 80% confluence, they were detached using 0.25% Trypsin-EDTA (GIBCO, USA) and re-plated at a density of 1×10^4 cells/cm^2 for expansion. Surface marker identification of the cultured MSCs was performed with a FACScan (Becton Dickinson, Franklin Lakes, NJ, USA) by fluorescein isothiocyanate (FITC)-labeled monoclonal antibody staining to CD45 (Antigenix America, Huntington Station, NY, USA) and phycoerythrin (PE)-conjugated antibodies against CD29 (VMRD, Pullman, WA, USA), CD44 and CD90 (Becton Dickinson). Isotypic antibodies served as the control.

MSCs were labeled with Feridex (Advanced Magnetics, Cambridge, MA, USA), as previously described[5]. Briefly, the polyamine poly-l-lysine (PLL) hydrobromide (Sigma, St Louis, MO, USA) was used as the transfection agent. A stock solution of PLL (1.5 mg/mL) was added to DMEM at a dilution of 1:1000 and mixed with Feridex (50 μg/mL) for 60 min at room temperature on a rotating shaker. MSCs of passage 5 were added to the culture medium that contained the Feridex-PLL complex, so that the final concentrations of Feridex and PLL were 25 μg/mL and 0.75 μg/mL, respectively. The cells were placed into six-well plates (Corning, NY, USA) overnight at 37℃ in a 95% air/5% CO_2 atmosphere.

Prussian blue staining

After being incubated overnight with the Feridex-PLL complex, the MSCs were washed three times to remove excessive contrast agent. For Prussian blue staining, which indicates the presence of iron, the coverslip samples were fixed with 4% paraformaldehyde for 30 min, washed, incubated for another 30 min with 2% potassium ferrocyanide in 6% hydrochloric acid, washed again, and counterstained with nuclear fast red.

Cell viability assay

Firstly, MSCs were inoculated in 96-well plates at 1×10^4 cells per well at 37℃ in a 95% air/5% CO_2 atmosphere. Twenty-four hours later, final concentrations of Feridex in the Feridex-PLL complex (25, 50, 100 and 200 μg/mL) were added to each well with 11 other duplicates and incubated overnight. The remaining cells, which were not labeled with the complex and served as control cells, were kept under identical conditions. The magnetically labeled and non-labeled cells were then maintained in fresh culture medium for 2 d and washed twice. Ten microliters of cholecystokinin octapeptide (CCK-8; Dojindo Laboratories, Kumamoto, Japan) was added per well for 4 h. The absorbance was then measured at a wavelength of 450 nm.

Swine model of acute liver injury

Under general anesthesia with mechanical ventilation *via* an endotracheal tube, animals were administered a dose of 0.5 g/kg of D-galactosamine (D-Gal; Sigma) dissolved in 5% glucose solution, *via* the external jugular vein. Venous blood samples were drawn 6, 12 and 24 h after the operation for biochemical analysis.

Intraportal transplantation of MSCs

Animals were randomly assigned to either control ($n = 4$) or experimental (liver injured) groups ($n = 6$). The abdomens of the liver-injured animals were opened to expose the portal vein, and approximately 1×10^7 labeled MSCs suspended in 2 mL DMEM were slowly injected into the portal vein. A 30-gauge needle was used for the procedure. The pinhole at the injection site was pressed for hemostasis. Thereafter, the laparotomy incision was enclosed

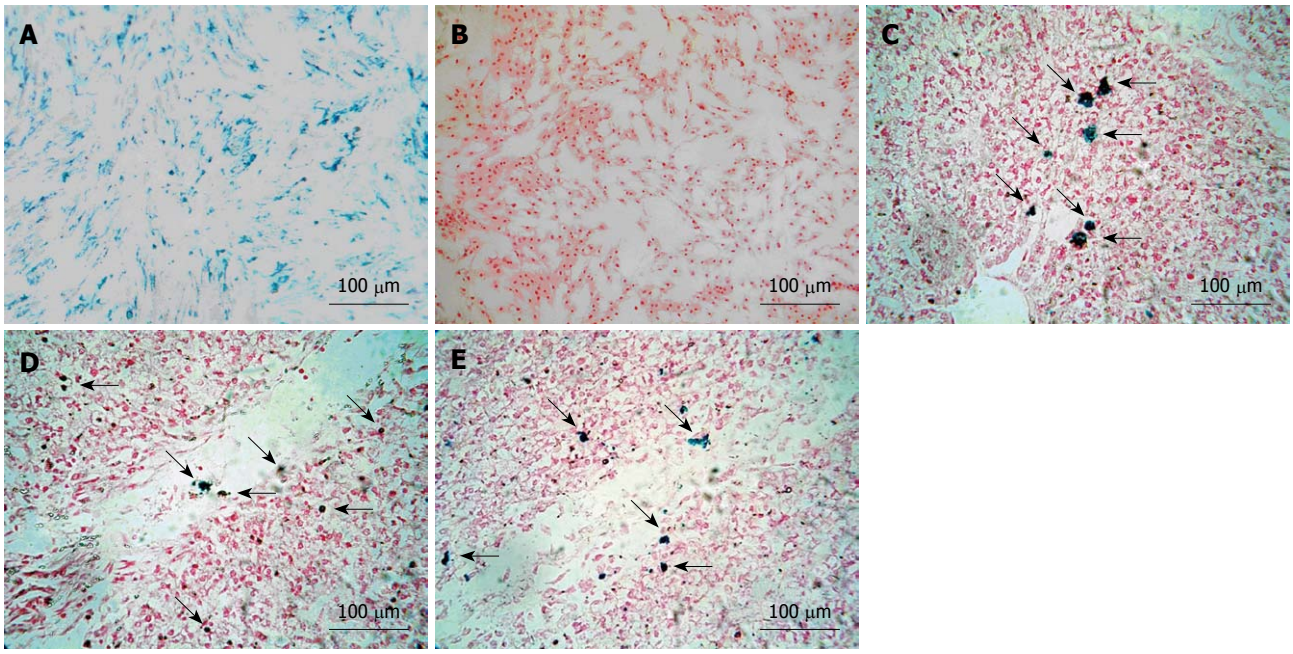

Figure 1 Characterization of labeled mesenchymal stem cells. A: Almost 100% of labeled mesenchymal stem cells (MSCs) were positive for Prussian blue staining (magnification 100 ×); B: No blue particles were observed in unlabeled group (magnification 100 ×); C-E: Prussian blue staining for liver tissue slicing displayed several blue-positive cells scattering in and around sinusoids on day 3 and 7, and the experimental group at the endpoint of the experiment (magnification 100 ×). Arrows indicate Prussian blue positive MSCs.

in layers. The control group underwent the identical procedure except that the injected cells were unlabeled.

MRI and data acquisition

Animals underwent MRI of the liver immediately before, and 3, 7 and 14 d after injection of cells. MRI was performed with a 1.5-T imaging device (Philips Medical Systems, Eindhoven, the Netherlands). The pig was anesthetized and placed supine on a plastic flat plate. The scanning sequence was as follows: (1) SE: T1WI, TR 120 s, TE 14 ms; (2) FSE: T2WI, TR 3000 ms, TE 96 ms; and (3) FFE: T2*WI, TR 485 ms, TE 14.0 ms, flip angle, 18°.

Histological assessment

Two weeks after cell transplantation, animals were sacrificed for histological examination. Liver tissues taken from both the control and experimental groups were fixed with 4% paraformaldehyde, embedded in paraffin, cut into 5-mm sections and stained with hematoxylin and eosin (HE) as well as Prussian blue for examination under a light microscope.

Statistical analysis

Data were shown as mean ± SE. The two-tailed unpaired Student's *t* test was used to evaluate the statistical significance of differences which was set with a *P* value less than 0.05.

RESULTS

MSC phenotype

Twenty-four hours after first seeding, MSCs could be seen in newly formed colonies. Observed under the microscope, the MSCs rapidly grew fibroblast-like cells with a single nucleus. After the first passage, they looked like spindles or asters with a slim body. At passage 5, however, most of the miscellaneous cells were eliminated, and the remaining uniform fibroblast-like cells were MSCs. The expression of different cell surface markers, including CD29, CD44, CD45 and CD90, of MSCs from passage 5 was determined by flow cytometry; the results of which showed that > 90% of MSCs of passage 5 were positive for CD29, CD44 and CD90, but negative for CD45 (data not shown).

Characterization of labeled MSCs

MSCs stained with Prussian blue showed results (blue particles) in all labeled cells. The labeling rate was approximately 100%, which was calculated under a light microscope *via* counting the numbers of positive cells in five random fields (Figure 1A). In contrast, no blue particles were observed in the unlabeled group (Figure 1B).

Proliferation of labeled MSCs

The growth curve of CCK-8 with Feridex-PLL labeled MSCs showed that the cellular proliferation of the 25 and 50 μg/mL subgroups were not significantly influenced by different concentration ($P > 0.05$). Figure 2 shows that MSCs labeled with higher concentrations of complex were somewhat inhibited in proliferation, which indicated that < 50 μg/mL Feridex-PLL would be suitable for MSC labeling in future transplantation.

Establishment of acute liver injury model

Acute liver injury was effectively induced in all animals. Serum alanine aminotransferase (ALT), aspartate amino-

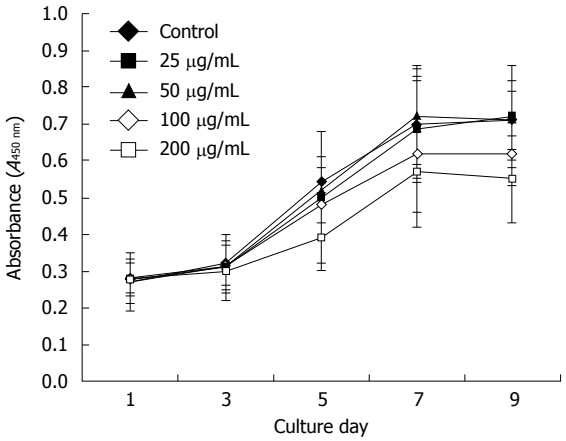

Figure 2 Growth curve of CCK-8 with Feridex-poly-l-lysine-labeled mesenchymal stem cells. Cellular proliferation of 25 and 50 μg/mL subgroups were not significantly influenced ($P > 0.05$). Mesenchymal stem cells (MSCs) labeled with higher concentrations of complex were somewhat inhibited, which indicated that < 50 μg/mL Feridex-poly-l-lysine would be suitable for MSC labeling.

transferase (AST) and bilirubin levels were all significantly and progressively elevated 6 h after D-Gal induction (Table 1, $P < 0.05$). The porcine model clinically presented listlessness, loss of appetite and xanthochromia. Liver tissue samples from the injured model group after 24 h injection of D-Gal demonstrated severe hepatic necrosis in 60%-70% of the lobule, sinusoidal congestion, vacuolization, trabecular fragmentation, and granulocytic infiltration (Figure 3). Twenty-four hours after administration of D-Gal, the animals were ready for transplantation (Table 1).

MR tracking of magnetically labeled MSCs

Signal intensity decreased 6 h after intrahepatic transplantation of labeled MSCs on the FFE sequence, but gradually approached close to normal on day 14 (Figure 4). For the control group, there was no visible difference at each time point after transplantation compared with before.

Histological demonstrations

The Prussian blue staining demonstrated several blue-positive cells scattered in and around the sinusoids in the experimental group on day 3 and 7 and the endpoint of experiment (Figure 1C-E), which indicated the presence of the magnetically labeled MSCs transplanted into the liver.

DISCUSSION

Bone marrow-derived MSCs are multipotent adult stem cells of mesodermal origin with the potential for self renewal. Because MSCs have the ability to differentiate into cells of multiple organs/systems such as hepatocytes, osteoblasts, chondrocytes, adipocytes and myocytes under appropriate stimuli[1,2,6-9], they have generated considerable interest for their potential use in regenerative medicine and tissue engineering. Given the ease of their isolation, extensive expansion rate and differentiation potential, as well as their immunosuppressive properties, MSCs may be suitable candidates for seed cells for hepatocyte transplantation, in addition to being potential carrier cells for gene therapy[10], which holds a promising future in the treatment of acute or chronic liver failure and metabolic liver diseases. Despite their important potential, however, the detailed processes involved in MSC implantation, migration, and differentiation in the liver remain to be elucidated.

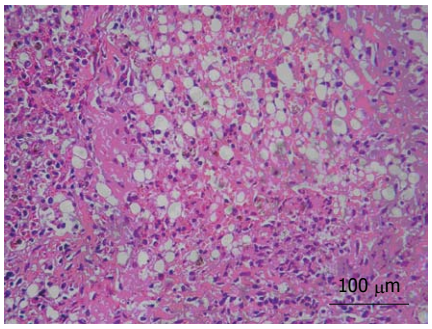

Figure 3 Histology of acutely injured liver tissue. Liver tissue samples from the injured model group after 24 h injection of D-galactosamine demonstrated severe hepatic necrosis of 60%-70% of the lobule, sinusoidal congestion, vacuolization, trabecular fragmentation, and granulocytic infiltration of the portal space and septa (magnification 100 ×).

Table 1 Biochemical parameters before and after the injection of D-galactosamine

	Pre-injection	6 h after injection	24 h after injection
ALT (U/L)	31.2 ± 2.6	87.5 ± 13.2	181.9 ± 12.8
AST (U/L)	28.9 ± 3.8	134.0 ± 7.8	564.8 ± 89.7
TBIL (μmol/L)	3.2 ± 0.45	26.7 ± 3.2	43.0 ± 2.9

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; TBIL: Total bilirubin.

Previous tissue slicing experiments have been unable to monitor the dynamic changes of transplanted cells in the living body. Accordingly, a noninvasive and living labeling technique is needed with respect to MSC intrahepatic transplantation. With the advent of molecular imaging technologies, *in vivo* real-time tracking and detecting the fate of transplanted stem cells may become a reality[3,11,12]. Cells for transplantation have been labeled with MR contrast agents since the beginning of the 1990s. In this regard, MRI appears most promising for dynamically monitoring *in vivo* cell migration after transplantation, due to its well known properties of relative long-term imaging, high spatial resolution, and sharp contrast[13]. Currently, SPIO MRI contrast agents have been most widely used for tracking transplanted cells in various organs because of their strong signal attenuation properties[14-18]. In particular, dextran-coated SPIO nanoparticles have been approved by the US Food and Drug Administration for use in hepatic reticuloendothelial cell imaging, and ultrasmall SPIOs are in phase Ⅲ clinical trials for use as blood pool agents or for use with lymphography[19-21]. However, such contrast agents cannot be used to label efficiently stem cells *in vitro* in their native unmodified form[22]. By conjugating antigen-specific internalizing monoclonal antibodies to the surface dextran coating, cells can be magnetically labeled

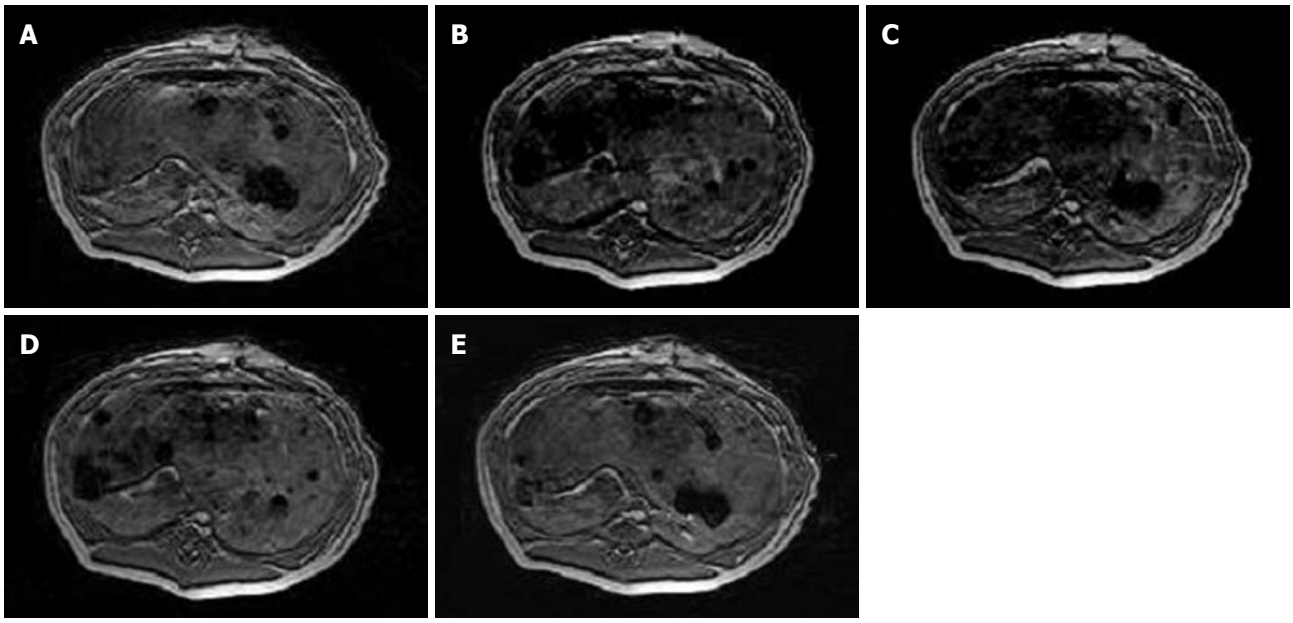

Figure 4 Magnetic resonance imaging of Feridex-poly-l-lysine labeled mesenchymal stem cells in the liver. A: No loss of signal in the liver before transplantation; B: Significant signal intensity loss was observed in labeled mesenchymal stem cells on FFE sequences 6 h after transplantation; C, D: Attenuation of signal loss appeared over time; E: T2*-weighted images gradually approached to the normal level at the endpoint of the study.

during their normal expansion in culture medium. In the present study, we used PLL as a transfection agent to magnetically label MSCs *in vitro* by the establishment of a Feridex-PLL complex through electrostatic interactions. This encourages cellular membrane endocytosis and transportation of Feridex into endosomes without requiring novel synthesis or covalent binding of proteins or antibodies to the dextran coating. Our results indicated that the labeling efficiency in our study approximated to 100% as expected. Subsequent analysis of the inhibitory effects of different concentrations of Feridex-PLL complex on MSCs revealed < 50 μg/mL Feridex was a relatively safe and effective dose for MSC labeling, which was suitable for transplantation study.

So far, most studies concerning MRI of grafted stem cells have been applied to animal brains, spines and hearts[17,23,24]. MRI techniques offer the possibility of tracking labeled cells *in vivo* noninvasively and repeatedly during extended study periods. The potential of MRI for future clinical interventions within the realm of regenerative cell therapy has been elegantly demonstrated in previous studies, and MRI fluoroscopy has been used to guide the delivery of MR-labeled adult stem cells into damaged organs. In our study, migration and retention of porcine MSCs after intraportal transplantation were demonstrated by using *in vivo* MRI. Significant signal intensity loss was observed in labeled MSCs on FFE sequences 6 h after transplantation. Thereafter, it gradually approached normal levels on day 14. The loss of signal could be attributed to either biodegradation of the contrast agent, the process of cellular division, or cellular migration to neighboring organs. To confirm the long-term results, Prussian blue staining was performed to demonstrate positive cells in liver tissue slices at the endpoint of the experiment. Furthermore, the dispersed distribution of labeled MSCs confirmed that the acute injured liver model may offer an ideal microenvironment for cell recruitment and implantation.

In summary, the present study incubated porcine MSCs with Feridex-PLL complex *in vitro*, and *in vivo* real-time tracking and detecting of magnetically labeled MSCs were manipulated by MRI in models of acute liver injury. Future research will focus on the optimized numbers of transplanted MSCs, distribution beyond injured liver, as well as safety and therapeutic effects concerning liver regeneration after MSC intraportal transplantation. In addition, our newly established co-culture system for hepatocytes and MSCs for cell transplantation and bioartificial liver devices should also be monitored[25].

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COMMENTS

Background

In recent years, cell transplantation has had the advantages of lower cost, lower risk, and simpler manipulation of the procedure compared with orthotopic liver transplantation. Autologous cell transplantation helps prevent immunological rejection, which is always a problem for orthotopic liver transplantation. Moreover, a large body of evidence has suggested that mesenchymal stem cells (MSCs) differentiate into liver-like cells with partial hepatic functions under appropriate environmental conditions *in vivo* and *in vitro*. Therefore, MSCs could be regarded as seeding cells for transplantation in relation to liver diseases.

Research frontiers

One of the major issues in liver cell transplantation is monitoring migration, distribution, and differentiation of the transplanted cells. Present tagging methods require *in vitro* preparation and examination of histological materials, which are unsuitable for noninvasive and repeated monitoring of *in vivo* transplanted cells under clinical conditions. Therefore, more recent research

has focused on *in vivo* real-time tracking and detecting the fate of transplanted cells by using appropriate imaging technologies.

Innovations and breakthroughs

The authors sought to label MSCs *in vitro* with super paramagnetic iron oxide (SPIO) and to monitor the labeled cells with magnetic resonance imaging (MRI). Through this research, they found a new invasive and repeatable monitoring method.

Applications

This method of labeling and monitoring cells could be applied to research in cell transplantation. In future, it could be considered as an invasive and repeatable monitoring method for clinical cell transplantation.

Terminology

SPIO is an MRI contrast agent that shows a high signal during imaging.

Peer review

In this study, authors successfully showed the use of swine mesenchymal stem cells in an acute liver injury model. The study should be considered as a brief report as it is a nice piece of information.

REFERENCES

1 **Shi XL**, Mao L, Xu BY, Xie T, Zhu ZH, Chen JH, Li L, Ding YT. Optimization of an effective directed differentiation medium for differentiating mouse bone marrow mesenchymal stem cells into hepatocytes in vitro. *Cell Biol Int* 2008; **32**: 959-965

2 **Sgodda M**, Aurich H, Kleist S, Aurich I, König S, Dollinger MM, Fleig WE, Christ B. Hepatocyte differentiation of mesenchymal stem cells from rat peritoneal adipose tissue in vitro and in vivo. *Exp Cell Res* 2007; **313**: 2875-2886

3 **Weissleder R**. Molecular imaging: exploring the next frontier. *Radiology* 1999; **212**: 609-614

4 **Nakamura Y**, Wang X, Xu C, Asakura A, Yoshiyama M, From AH, Zhang J. Xenotransplantation of long-term-cultured swine bone marrow-derived mesenchymal stem cells. *Stem Cells* 2007; **25**: 612-620

5 **Arbab AS**, Bashaw LA, Miller BR, Jordan EK, Lewis BK, Kalish H, Frank JA. Characterization of biophysical and metabolic properties of cells labeled with superparamagnetic iron oxide nanoparticles and transfection agent for cellular MR imaging. *Radiology* 2003; **229**: 838-846

6 **Heino TJ**, Hentunen TA. Differentiation of osteoblasts and osteocytes from mesenchymal stem cells. *Curr Stem Cell Res Ther* 2008; **3**: 131-145

7 **Yokoyama M**, Miwa H, Maeda S, Wakitani S, Takagi M. Influence of fetal calf serum on differentiation of mesenchymal stem cells to chondrocytes during expansion. *J Biosci Bioeng* 2008; **106**: 46-50

8 **Scavo LM**, Karas M, Murray M, Leroith D. Insulin-like growth factor-I stimulates both cell growth and lipogenesis during differentiation of human mesenchymal stem cells into adipocytes. *J Clin Endocrinol Metab* 2004; **89**: 3543-3553

9 **Li X**, Yu X, Lin Q, Deng C, Shan Z, Yang M, Lin S. Bone marrow mesenchymal stem cells differentiate into functional cardiac phenotypes by cardiac microenvironment. *J Mol Cell Cardiol* 2007; **42**: 295-303

10 **Javazon EH**, Beggs KJ, Flake AW. Mesenchymal stem cells: paradoxes of passaging. *Exp Hematol* 2004; **32**: 414-425

11 **Swijnenburg RJ**, van der Bogt KE, Sheikh AY, Cao F, Wu JC. Clinical hurdles for the transplantation of cardiomyocytes derived from human embryonic stem cells: role of molecular imaging. *Curr Opin Biotechnol* 2007; **18**: 38-45

12 **Shah K**, Weissleder R. Molecular optical imaging: applications leading to the development of present day therapeutics. *NeuroRx* 2005; **2**: 215-225

13 **Delikatny EJ**, Poptani H. MR techniques for in vivo molecular and cellular imaging. *Radiol Clin North Am* 2005; **43**: 205-220

14 **Engberink RD**, Blezer EL, Hoff EI, van der Pol SM, van der Toorn A, Dijkhuizen RM, de Vries HE. MRI of monocyte infiltration in an animal model of neuroinflammation using SPIO-labeled monocytes or free USPIO. *J Cereb Blood Flow Metab* 2008; **28**: 841-851

15 **Lee SM**, Lee SH, Kang HY, Baek SY, Kim SM, Shin MJ. Assessment of musculoskeletal infection in rats to determine usefulness of SPIO-enhanced MRI. *AJR Am J Roentgenol* 2007; **189**: 542-548

16 **Oude Engberink RD**, van der Pol SM, Döpp EA, de Vries HE, Blezer EL. Comparison of SPIO and USPIO for in vitro labeling of human monocytes: MR detection and cell function. *Radiology* 2007; **243**: 467-474

17 **Fukuda Y**, Ando K, Ishikura R, Kotoura N, Tsuda N, Kato N, Yoshiya S, Nakao N. Superparamagnetic iron oxide (SPIO) MRI contrast agent for bone marrow imaging: differentiating bone metastasis and osteomyelitis. *Magn Reson Med Sci* 2006; **5**: 191-196

18 **Kim SH**, Lee JM, Han JK, Lee JY, Kang WJ, Jang JY, Shin KS, Cho KC, Choi BI. MDCT and superparamagnetic iron oxide (SPIO)-enhanced MR findings of intrapancreatic accessory spleen in seven patients. *Eur Radiol* 2006; **16**: 1887-1897

19 **Harisinghani MG**, Saini S, Weissleder R, Hahn PF, Yantiss RK, Tempany C, Wood BJ, Mueller PR. MR lymphangiography using ultrasmall superparamagnetic iron oxide in patients with primary abdominal and pelvic malignancies: radiographic-pathologic correlation. *AJR Am J Roentgenol* 1999; **172**: 1347-1351

20 **Gandhi SN**, Brown MA, Wong JG, Aguirre DA, Sirlin CB. MR contrast agents for liver imaging: what, when, how. *Radiographics* 2006; **26**: 1621-1636

21 **Mack MG**, Balzer JO, Straub R, Eichler K, Vogl TJ. Superparamagnetic iron oxide-enhanced MR imaging of head and neck lymph nodes. *Radiology* 2002; **222**: 239-244

22 **Lewin M**, Carlesso N, Tung CH, Tang XW, Cory D, Scadden DT, Weissleder R. Tat peptide-derivatized magnetic nanoparticles allow in vivo tracking and recovery of progenitor cells. *Nat Biotechnol* 2000; **18**: 410-414

23 **Wu X**, Hu J, Zhou L, Mao Y, Yang B, Gao L, Xie R, Xu F, Zhang D, Liu J, Zhu J. In vivo tracking of superparamagnetic iron oxide nanoparticle-labeled mesenchymal stem cell tropism to malignant gliomas using magnetic resonance imaging. Laboratory investigation. *J Neurosurg* 2008; **108**: 320-329

24 **He G**, Zhang H, Wei H, Wang Y, Zhang X, Tang Y, Wei Y, Hu S. In vivo imaging of bone marrow mesenchymal stem cells transplanted into myocardium using magnetic resonance imaging: a novel method to trace the transplanted cells. *Int J Cardiol* 2007; **114**: 4-10

25 **Gu J**, Shi X, Zhang Y, Chu X, Hang H, Ding Y. Establishment of a three-dimensional co-culture system by porcine hepatocytes and bone marrow mesenchymal stem cells in vitro. *Hepatol Res* 2009; **39**: 398-407

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BRIEF ARTICLE

ICC density predicts bacterial overgrowth in a rat model of post-infectious IBS

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Abstract

AIM: To investigate the interstitial cells of Cajal (ICC) number using a new rat model.

METHODS: Sprague-Dawley rats were assigned to two groups. The first group received gavage with *Campylobacter jejuni* (*C. jejuni*) 81-176. The second group was gavaged with placebo. Three months after clearance of *Campylobacter* from the stool, precise segments of duodenum, jejunum, and ileum were ligated in self-contained loops of bowel that were preserved in anaerobic bags. Deep muscular plexus ICC (DMP-ICC) were quantified by two blinded readers assessing the tissue in a random, coded order. The number of ICC per villus was compared among controls, *Campylobacter* recovered rats without small intestinal bacterial overgrowth (SIBO), and *Campylobacter* recovered rats with SIBO.

RESULTS: Three months after recovery, 27% of rats gavaged with *C. jejuni* had SIBO. The rats with SIBO had a lower number of DMP-ICC than controls in the jejunum and ileum. Additionally there appeared to be a density threshold of 0.12 DMP-ICC/villus that was associated with SIBO. If ileal density of DMP-ICC was < 0.12 ICC/villus, 54% of rats had SIBO compared to 9% among ileal sections with > 0.12 ($P < 0.05$). If the density of ICC was < 0.12 DMP-ICC/villus in more than one location of the bowel, 88% of these had SIBO compared to 6% in those who did not ($P < 0.001$).

CONCLUSION: In this post-infectious rat model, the development of SIBO appears to be associated with a reduction in DMP-ICC. Further study of this rat model might help understand the pathophysiology of post-infectious irritable bowel syndrome.

Key words: Post-infectious irritable bowel syndrome; Bacterial overgrowth; Interstitial cells of Cajal; Campylobacter

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INTRODUCTION

A recent series of studies suggested a link between small

intestinal bacterial overgrowth (SIBO) and irritable bowel syndrome (IBS)[1,2]. The latest of these is a study that compared jejunal cultures between IBS patients and controls[3]. In that study, the number of coliform bacteria was higher in the small bowel of IBS compared to healthy controls.

Another potential bacterial pathogenesis in IBS is related to the development of IBS symptoms after acute gastroenteritis [post-infectious IBS (PI-IBS)]. In a recent meta-analysis, almost 10% of normal subjects developed IBS after an incident of bacterial gastroenteritis[4]. Initial research in this area focused on possible inflammatory events, as suggested by increased lymphocyte counts in the rectal mucosa[5-7]. Recent evidence has linked post-infectious events to the development of SIBO in a rat model of PI-IBS[8]. In this model, rats exposed to *Campylobacter jejuni* (*C. jejuni*) have persistent altered stool form that is linked to the development of SIBO in 27% of rats, based on quantitative polymerase chain reaction (PCR). This finding in rats suggests that, at least in the case of *C. jejuni*, a possible mechanism for PI-IBS could be the development of SIBO. How SIBO develops in this model is not known. In humans, it has been speculated that SIBO in IBS patients is related to a reduced number of fasting migrating motor complexes[9]. This has been a recognized cause of SIBO since 1977[10].

The important role of interstitial cells of Cajal (ICC) in gastrointestinal physiology has been elucidated over the past 10-20 years. ICC are required for normal intestinal motility. Their role as intestinal pacemakers has been established in a number of model systems[11-13]. Labeling ICC with anti-Kit antibodies provides an opportunity to evaluate ICC of gastrointestinal muscles in patients with various motility disorders. Reduced numbers or loss of ICC is associated with several motor dysfunctions, including achalasia, intestinal pseudoobstruction, infantile pyloric stenosis, diabetic gastroparesis, ulcerative colitis, and slow-transit constipation[14-19]. Loss of ICC also interferes with electrical pacemaker activity, slow-wave propagation, and gastrointestinal motor neurotransmission[12,13,20,21].

Given the significant role of ICC in modulating the neuromuscular activity of the gut, we sought to investigate whether the development of SIBO in the rat model infected with *C. jejuni* is associated with reduction in intestinal ICC.

MATERIALS AND METHODS

Development of post-infectious rats

After confirming no pre-existing presence of *C. jejuni* in their stools, 66 outbred Sprague-Dawley rats were randomized (in a 1:1 ratio) into two groups. One group was gavaged with a 1 mL solution containing *C. jejuni* 81-176 at 10^8 CFU/mL (C+ rats) in Brucella broth. The control rats were gavaged with an identical solution without *C. jejuni* (C- rats).

Following gavage, all the rats were housed at two per cage. However, the rats receiving *Campylobacter* (C+) were housed separately from the control (C-) rats to avoid the possibility of cross contamination of the *C. jejuni* infection between the two groups.

In the first 3 d after gavage, stool was collected from both groups to verify that intestinal colonization in C+ rats had occurred. It was also used to confirm the absence of infection in C- rats. Stool was later obtained in the C+ group on days seven and 14 after infection and then every 2 wk until two consecutive negative cultures for *C. jejuni* were confirmed.

After 90% of the C+ rats no longer had detectable *C. jejuni* in the stool, they were considered to be in the post-infectious time period. At this point, they were housed for three additional months. During this time, both groups were treated equally with respect to food, water, and environment. In the 3 d just prior to euthanasia (at 3 mo into the post-infectious period) stool was collected from each rat and graded blindly according to an *a priori* stool form grading score. This score was based on whether or not there was loose or normal stool in a blinded evaluation. Any loose stool was considered abnormal. The stool was also cultured to determine if there was any lingering case of *Campylobacter* (C+).

Campylobacter gavage

The *C. jejuni* 81-176 strain used in the gavage of the rats was obtained from freezer stocks, plated on selective media, and incubated for 36-48 h under microaerophilic conditions at 42℃ to create a bacterial lawn. This lawn was then harvested from these plates and suspended in Brucella broth. The concentration of bacteria was estimated spectrophotometrically and confirmed *via* serial dilution and plating on selective media. In the 30 min prior to *Campylobacter* gavage, rats were gavaged with a 1 mL solution of 5% sodium bicarbonate using a ball-tipped inoculating needle. This was done to neutralize gastric acid to increase the likelihood of intestinal colonization of the pathogen. Subsequently, a 1 mL suspension of *C. jejuni* in Brucella broth (5×10^8 CFU/mL) was administered by gavage.

Bowel sampling

Three months after clearance of *Campylobacter* from the stool, fresh stool was obtained from all rats. This was used to determine the presence or absence of prolonged *C. jejuni* colonization. Stool was also qualitatively evaluated for stool form as described above.

Rats were then euthanized. Immediately following euthanasia, a laparotomy was performed whereby precisely determined segments of the ileum, jejunum, and duodenum were obtained, as previously described[8]. The first 2 cm segment at each location was a ligated self-contained loop of bowel. Sutures were placed on either side to prevent exposure to air. Samples were kept at 4℃ in an anaerobic bag for transport. These self-contained loops were then used to extract bacterial contents for the determination of bacterial number using quantitative

PCR, as previously described[8]. The quantity of bacteria in these segments was compared between control rats and rats 3 mo after recovery from *Campylobacter*. SIBO was considered to be present when bacterial count in the *Campylobacter* treated rats exceeded two standard deviations above the mean count found in the control group.

In each rat, a 2 cm segment of small bowel immediately adjacent to the closed loop of small bowel was resected and sent for bacterial quantitation. This second piece of bowel was opened longitudinally along the mesenteric border and placed in a solution of 10% formalin (VWR, West Chester, PA). After paraffin and mounting, the tissue was stained.

Immunohistochemistry

Rats were then divided into three groups based on the presence or absence of *Campylobacter* and SIBO. The three groups were a random selection of eight control rats (C-), eight *Campylobacter*-infected rats that were found to have bacterial overgrowth (C+/SIBO+), and eight randomly selected rats that received *Campylobacter* gavage but did not develop bacterial overgrowth (C+/SIBO-). Sections from each paraffin block were stained immunohistochemically using Polyclonal Rabbit Anti-Human CD117, c-kit (Dako-Cytomation, Carpinteria, CA). The positive control used to test the quality of the stain was a c-kit positive gastrointestinal stromal tumor. ICC were quantified by two blinded readers assessing the tissue in a random, coded order. The number of ICC was evaluated in the region of the deep muscle plexus ICC (DMP-ICC) according to the number of villi. The number of DMP-ICC per villus was compared among controls, C+/SIBO-, and C+/SIBO+.

Statistical analysis

Statistical comparisons for the number of DMP-ICC per villus were made by one-way analysis of variance among three groups and differences from controls was further analyzed using the Wilcoxon Rank-Sum Test. To compare the density threshold of ICC per villus, a Fisher's exact test was used. A P value of <0.05 was considered significant.

RESULTS

Campylobacter infection

Out of 66 rats used in the study, none had stool culture demonstrating *C. jejuni* before gavage. Of these rats, 33 were gavaged with vehicle (C-) and 33 rats received approximately 5×10^8 CFU *C. jejuni* 81-176 (C+). Six rats died of trauma secondary to gavage (three in each group). Among the remaining 30 rats that received *C. jejuni*, all had stool cultures that confirmed intestinal colonization by *C. jejuni*, and all but one rat cleared this colonization by 14 d.

As previously reported[8], C- rats were then used to determine the normal range of flora in the duodenum, jejunum, and ileum. Using these control data, eight C+ rats (27%) were found to have SIBO and were designated C+/SIBO+.

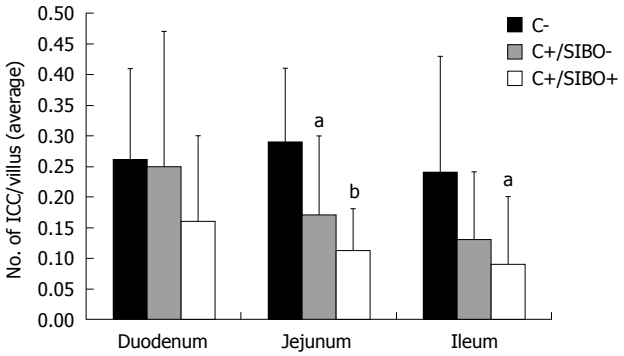

Figure 1 The number of interstitial cells of Cajal per villus in the duodenum, jejunum, and ileum. C-: Rats that received vehicle gavage; C+/SIBO-: Rats gavaged with *Campylobacter* but which did not develop small intestinal bacterial overgrowth (SIBO); C+/SIBO+: Rats gavaged with *Campylobacter* that later developed SIBO. $^aP < 0.05$, $^bP < 0.001$ *vs* control.

The number of DMP-ICC per villus

The number of DMP-ICC per villus was determined in C-, C+/SIBO-, and C+/SIBO+ rats. As shown in Figure 1, the rats with SIBO had the lowest number of DMP-ICC. This was more obvious in the jejunum and ileum than in the duodenum. Although there was a reduction of ICC in the C+/SIBO- group, this was not as great as was seen in the C+/SIBO+ group. Figure 2 shows representative examples of ileal biopsies in the C-, C+/SIBO-, and C+/SIBO+ groups, respectively. There was a reduced number of CD117 stained cells in the deep muscular plexus in the C+/SIBO+ group.

Density threshold of DMP-ICC

The number of DMP-ICC was then used to determine the level of ICC compared to SIBO. The data suggested that rats with < 0.12 ICC/villus were most likely to have SIBO. In fact, 54% of rats with a density < 0.12 ICC/villus in the ileum had SIBO compared to 9% in which DMP-ICC density was ⩾ 0.12/villus ($P < 0.05$). Using all levels of bowel, the density threshold was even more relevant. If the density of DMP-ICC/villus was < 0.12/villus in more than one of the three bowel segments, 88% had SIBO compared to 6% if the figure was ⩾ 0.12/villus ($P < 0.001$).

Differential effects of campylobacter on ICC

When the number of DMP-ICC was noted by segment of bowel according to whether rats received vehicle or *Campylobacter*, a significant difference was seen (Figure 3). There was a graded affect on DMP-ICC population, such that the greatest effect on reduction in DMP-ICC was in the distal small bowel. Reduction was also seen in the jejunum and ileum. This contrasted to the control group, whereby DMP-ICC density was uniform from proximal to distal small bowel.

DISCUSSION

In a previous study, we demonstrated that in a post-infectious rat model, altered bowel function persisted in

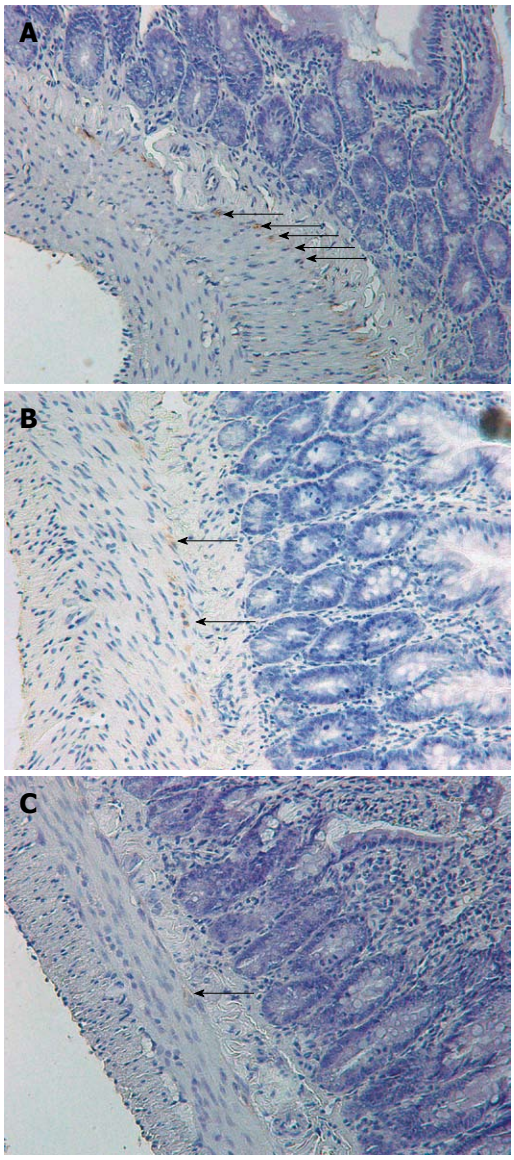

Figure 2 Interstitial cells of Cajal (arrows) in the ileum stained with CD117 (magnification 40 ×). A: Control rat; B: Rat exposed to *Campylobacter* that did not develop small intestinal bacterial overgrowth (SIBO), demonstrating persistent CD117 staining of interstitial cells of Cajal (ICC) cells; C: Rat exposed to *Campylobacter* that developed SIBO, demonstrating a reduction in CD117 staining of ICC cells. In this case, the staining is slight and the arrow indicates a "possible" stained cell.

a large number of rats even 3 mo beyond the clearance of the infection[8]. 27% of rats were found to have SIBO by quantitative PCR. SIBO correlated with those animals that had the most altered stool form. In this study, we attempted to further understand how SIBO could develop in this post-infectious rat model by studying the ICC. We found that long after *Campylobacter* infection had cleared, there was an apparent reduction in the number of DMP-ICC. This reduction appeared most evident in rats that developed SIBO after clearing *Campylobacter*. Furthermore, when the DMP-ICC density is less than 0.12/villus, SIBO is predicted to occur.

Six to seventeen percent of IBS patients believe their symptoms began after acute gastroenteritis[22], and the

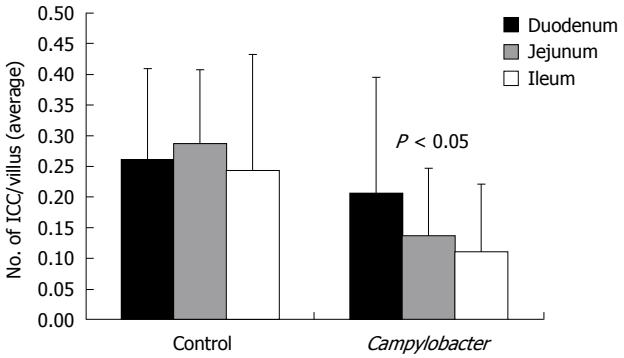

Figure 3 The effect of *Campylobacter* on interstitial cells of Cajal is greater in the distal small bowel (based on one-way analysis of variance).

incidence of PI-IBS following bacterial gastroenteritis is reported to be between 4%-31%[7,23-26]. In a 6-year follow up study, 57% of subjects thought to have developed PI-IBS still had altered bowel function consistent with IBS[27]. This growing body of literature on PI-IBS has led to two recent meta-analyses[4,28]. Both studies estimated that IBS has an incidence rate of about 10% after a case of acute gastroenteritis.

Two issues make PI-IBS very important in research on IBS pathophysiology. First, acute gastroenteritis is very common, and with a 10% rate of IBS development, this phenomenon might be very important in the development of IBS as a whole. Secondly, this is the first demonstration of a direct cause and effect relationship in the precipitation of IBS. How an acute gastrointestinal infectious process produces IBS in humans remains poorly understood, but some investigators have found residual inflammatory changes in the gut among patients with PI-IBS. These include increased numbers of lymphocytes, enteroendocrine cells, and mast cells in PI-IBS[5-7,26,29,30]. It is difficult to guarantee uniformity of population and pathogen in human studies of PI-IBS as they rely on studying a group of humans who emerge from an outbreak of infectious diarrhea. As a result, the study of inflammation and other factors in PI-IBS have been moving into animal models of PI-IBS.

One model that has been studied for some time is a *Trichinella spiralis* (*T. spiralis*)-infected mouse model. Beyond inflammatory changes, research in this model has looked at the long term effect on gastrointestinal motility. A major finding in this model was that, after resolution of the initial pathogen-related inflammatory response and elimination of the nematode from the gut, there was persistent intestinal neuromuscular dysfunction[31]. *T. spiralis*-induced mucosal inflammation led to prolonged effects on intestinal smooth muscle, and to colonic visceral hyperalgesia[32,33]. Although models such as this are vital to our understanding of PI-IBS, no model had been developed to investigate the more common human pathogens believed to cause the bulk of PI-IBS. These include *Campylobacter*, *Salmonella*, and *Shigella*, among others[34].

Two existing theories of bacterial events (post-infectious

and SIBO) now appear to be linked, based on a recent study suggesting that *C. jejuni* infection precipitates altered stool form and SIBO in a rat model[8]. How this occurs is currently unknown, although one study has demonstrated that acute *C. jejuni* infection alters intestinal myoelectric activity. In ileal segments of rabbits, alteration of action potential activity was seen in the small intestine infused intraluminally with *C. jejuni* and its cell-free filtrate[35]. In that study, the rabbits were monitored for only 24 h after exposure to *C. jejuni*. Unfortunately, no long term studies were done, and the cause of these effects was unknown.

An important cell in the control of gut motor function is the ICC. A growing list of animal models appears to support the notion that ICC are impaired in the presence of inflammation-induced changes in motor control. With acetic acid-induced inflammation, a reduction in resting membrane potential and the amplitude and duration of slow waves was related to the damage of the ICC in circular muscle cells in dogs[36]. In another study using the earlier described *T. spiralis*-model, damage to the structure of ICC networks within the region of the myenteric plexus was seen[32]. When jejunal inflammation was induced by *Nippostrongylus brasiliensis* in rats, changes in myenteric neurons, circular muscle cells, and ICC were observed[37].

In this study, we investigated whether the development of SIBO in the postinfectious rat model is related to the alteration of DMP-ICC populations, and whether this could in some way be linked to the development of IBS. In this study, *Campylobacter* gavaged rats were found to have a reduction of DMP-ICC density 3 mo after clearance of *Campylobacter*. More interesting was that the DMP-ICC density also corresponded to the development of SIBO. Rats with SIBO had the lowest DMP-ICC density. We could further evaluate a density threshold of DMP-ICC per villus which predicts SIBO. If the density of DMP-ICC/villus was not greater than 0.12 in more than one location among duodenum, jejunum, and ileum, 88% of the rats had SIBO. Although this finding does not examine the numerous contributions to gut motor function in addition to ICC, the possibility is raised that through some effects on ICC or neuromuscular mechanisms, acute gastroenteritis produces a long term effect that allows for the development of SIBO.

How *Campylobacter* affects DMP-ICC is unknown, but two possibilities include a toxin or a result of the initial acute inflammatory reaction. Although inflammation seems to be an obvious possibility, ICC's have a significant amount of "plasticity". Loss of ICC in pathological conditions does not always mean permanent injury. This "plasticity" of ICC was found in a murine model of partial bowel obstruction[38]. After the onset of the obstruction, hypertrophy of the smooth muscle layers and progressive loss of ICC orad to the site of obstruction were observed. Recovery of ICC and restoration of slow wave activity after removal of the obstruction were achieved within 30 d. In addition, injury to ICC due to inflammation is repaired in the course of time. Structural damage was observed in the network of ICC for 2 wk after *T. spiralis* infection. The structural changes were accompanied by aberrant pacemaker activity and abnormal slow waves. Sixty days after infection, motility and ICC recovered to normal values[32]. Unlike these two studies, we found a persistent decrease in the number of DMP-ICC 3 mo after clearance of infection. This was not due to any persistent *Campylobacter* colonization, as the pathogen was not detectable by culture in any location of the gastrointestinal tract. However, it is presumed that some event related to *Campylobacter* is responsible for this persistent reduction of DMP-ICC. This particular layer of ICC might be important. For example, in the W/W^{v} mouse, the DMP is intact and, despite loss of all other layers of ICC and loss of electrical slow wave, the migrating motor complex also remains intact[39]. Thus, DMP destruction might have an impact on mechanisms that protect against bacterial overgrowth.

An alternative explanation would be that SIBO is contributing to the reduction of ICC. Although not considered in this study, one means of determining this would be to provide antibiotic treatment to rats and count ICC after eradication of SIBO. The challenge with examining this concept is that it is difficult to identify SIBO in a live rat. This would make it difficult to know which animal should receive antibiotics.

It can be considered that ICC is just a marker of more global damage. In our study, smooth muscle was not evaluated. In a mouse model after acute nematode infection, altered neuromuscular function and long-lasting muscle contractility were noted[31,33]. The findings were considered as a model of PI-IBS. Further studies are needed to evaluate smooth muscle, toxin, and the effect of inflammation with *Campylobacter* infection.

In conclusion, rats with SIBO that developed 3 mo after *Campylobacter* gavage had a decreased number of ICC in the jejunum and ileum compared to control rats. Furthermore, the density threshold of ICC per villus appears to predict SIBO. These data suggest that a decrease in the number of ICC in the small intestine is implicated in the pathogenesis of PI-IBS. Elucidating which *Campylobacter*-related factor produces this decrease in the number of ICC may contribute greatly to our understanding of PI-IBS and lead to potential treatments for IBS.

COMMENTS

Background

There is a growing interest in understanding newly discovered bacterial mechanisms in the pathophysiology of functional bowel disease. These mechanisms might result innovative treatments for diseases such as irritable bowel syndrome (IBS).

Research frontiers

Two bacterial hypotheses have emerged in IBS. The first hypothesis is that IBS appears to develop in humans after an episode of acute gastroenteritis. The other bacterial hypothesis is that a proportion of patients who already have IBS appear to have small intestinal bacterial overgrowth (SIBO) and improve with antibiotic therapy.

Innovations and breakthroughs

Recently, a new animal model of post-infectious IBS has been developed on the basis of a common human pathogen, *Campylobacter jejuni* (*C. jejuni*). In

this model, IBS-like symptoms appear to develop in rats 3 mo after clearance of the initial infection. At this time, a proportion of these rats have bacterial overgrowth, based on quantitative polymerase chain reaction. These data now link acute gastroenteritis to the development of bacterial overgrowth and symptoms in an animal model.

Applications

This new animal model will facilitate the discovery of the cascade of events that lead to IBS and SIBO. One candidate in the cascade is likely to be an effect on the neuromuscular apparatus of the gut.

Terminology

Post-infectious IBS is the development of IBS after a self-limited infection of the intestine, such as acute gastroenteritis. Interstitial cells of Cajal (ICC) are nerve cells in the intestinal lining that are important for maintaining the function of the gut.

Peer review

In this manuscript, the authors investigate the potential role of a decreased number of ICC in causing SIBO after a *C. jejuni* infection in rats. The paper is well written and is pleasant to read. The experiments have been carefully designed and executed.

REFERENCES

1 **Pimentel M**, Chow EJ, Lin HC. Eradication of small intestinal bacterial overgrowth reduces symptoms of irritable bowel syndrome. *Am J Gastroenterol* 2000; **95**: 3503-3506
2 **Pimentel M**, Chow EJ, Lin HC. Normalization of lactulose breath testing correlates with symptom improvement in irritable bowel syndrome. a double-blind, randomized, placebo-controlled study. *Am J Gastroenterol* 2003; **98**: 412-419
3 **Posserud I**, Stotzer PO, Björnsson ES, Abrahamsson H, Simrén M. Small intestinal bacterial overgrowth in patients with irritable bowel syndrome. *Gut* 2007; **56**: 802-808
4 **Halvorson HA**, Schlett CD, Riddle MS. Postinfectious irritable bowel syndrome--a meta-analysis. *Am J Gastroenterol* 2006; **101**: 1894-1899; quiz 1942
5 **Gwee KA**, Leong YL, Graham C, McKendrick MW, Collins SM, Walters SJ, Underwood JE, Read NW. The role of psychological and biological factors in postinfective gut dysfunction. *Gut* 1999; **44**: 400-406
6 **Spiller RC**, Jenkins D, Thornley JP, Hebden JM, Wright T, Skinner M, Neal KR. Increased rectal mucosal enteroendocrine cells, T lymphocytes, and increased gut permeability following acute Campylobacter enteritis and in post-dysenteric irritable bowel syndrome. *Gut* 2000; **47**: 804-811
7 **Dunlop SP**, Jenkins D, Neal KR, Spiller RC. Relative importance of enterochromaffin cell hyperplasia, anxiety, and depression in postinfectious IBS. *Gastroenterology* 2003; **125**: 1651-1659
8 **Pimentel M**, Chatterjee S, Chang C, Low K, Song Y, Liu C, Morales W, Ali L, Lezcano S, Conklin J, Finegold S. A new rat model links two contemporary theories in irritable bowel syndrome. *Dig Dis Sci* 2008; **53**: 982-989
9 **Pimentel M**, Soffer EE, Chow EJ, Kong Y, Lin HC. Lower frequency of MMC is found in IBS subjects with abnormal lactulose breath test, suggesting bacterial overgrowth. *Dig Dis Sci* 2002; **47**: 2639-2643
10 **Vantrappen G**, Janssens J, Hellemans J, Ghoos Y. The interdigestive motor complex of normal subjects and patients with bacterial overgrowth of the small intestine. *J Clin Invest* 1977; **59**: 1158-1166
11 **Langton P**, Ward SM, Carl A, Norell MA, Sanders KM. Spontaneous electrical activity of interstitial cells of Cajal isolated from canine proximal colon. *Proc Natl Acad Sci USA* 1989; **86**: 7280-7284
12 **Ordög T**, Ward SM, Sanders KM. Interstitial cells of cajal generate electrical slow waves in the murine stomach. *J Physiol* 1999; **518** (Pt 1): 257-269
13 **Der-Silaphet T**, Malysz J, Hagel S, Larry Arsenault A, Huizinga JD. Interstitial cells of cajal direct normal propulsive contractile activity in the mouse small intestine. *Gastroenterology* 1998; **114**: 724-736
14 **Goldblum JR**, Whyte RI, Orringer MB, Appelman HD. Achalasia. A morphologic study of 42 resected specimens. *Am J Surg Pathol* 1994; **18**: 327-337
15 **Rumessen JJ**. Ultrastructure of interstitial cells of Cajal at the colonic submuscular border in patients with ulcerative colitis. *Gastroenterology* 1996; **111**: 1447-1455
16 **Streutker CJ**, Huizinga JD, Campbell F, Ho J, Riddell RH. Loss of CD117 (c-kit)- and CD34-positive ICC and associated CD34-positive fibroblasts defines a subpopulation of chronic intestinal pseudo-obstruction. *Am J Surg Pathol* 2003; **27**: 228-235
17 **Vanderwinden JM**, Liu H, De Laet MH, Vanderhaeghen JJ. Study of the interstitial cells of Cajal in infantile hypertrophic pyloric stenosis. *Gastroenterology* 1996; **111**: 279-288
18 **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
19 **Bassotti G**, Villanacci V, Maurer CA, Fisogni S, Di Fabio F, Cadei M, Morelli A, Panagiotis T, Cathomas G, Salerni B. The role of glial cells and apoptosis of enteric neurones in the neuropathology of intractable slow transit constipation. *Gut* 2006; **55**: 41-46
20 **Torihashi S**, Ward SM, Nishikawa S, Nishi K, Kobayashi S, Sanders KM. c-kit-dependent development of interstitial cells and electrical activity in the murine gastrointestinal tract. *Cell Tissue Res* 1995; **280**: 97-111
21 **Ward SM**, Harney SC, Bayguinov JR, McLaren GJ, Sanders KM. Development of electrical rhythmicity in the murine gastrointestinal tract is specifically encoded in the tunica muscularis. *J Physiol* 1997; **505** (Pt 1): 241-58
22 **Longstreth GF**, Hawkey CJ, Mayer EA, Jones RH, Naesdal J, Wilson IK, Peacock RA, Wiklund IK. Characteristics of patients with irritable bowel syndrome recruited from three sources: implications for clinical trials. *Aliment Pharmacol Ther* 2001; **15**: 959-964
23 **Gwee KA**, Graham JC, McKendrick MW, Collins SM, Marshall JS, Walters SJ, Read NW. Psychometric scores and persistence of irritable bowel after infectious diarrhoea. *Lancet* 1996; **347**: 150-153
24 **Neal KR**, Hebden J, Spiller R. Prevalence of gastrointestinal symptoms six months after bacterial gastroenteritis and risk factors for development of the irritable bowel syndrome: postal survey of patients. *BMJ* 1997; **314**: 779-782
25 **Thornley JP**, Jenkins D, Neal K, Wright T, Brough J, Spiller RC. Relationship of Campylobacter toxigenicity in vitro to the development of postinfectious irritable bowel syndrome. *J Infect Dis* 2001; **184**: 606-609
26 **Wang LH**, Fang XC, Pan GZ. Bacillary dysentery as a causative factor of irritable bowel syndrome and its pathogenesis. *Gut* 2004; **53**: 1096-1101
27 **Neal KR**, Barker L, Spiller RC. Prognosis in post-infective irritable bowel syndrome: a six year follow up study. *Gut* 2002; **51**: 410-413
28 **Thabane M**, Kottachchi DT, Marshall JK. Systematic review and meta-analysis: The incidence and prognosis of post-infectious irritable bowel syndrome. *Aliment Pharmacol Ther* 2007; **26**: 535-544
29 **O'Sullivan M**, Clayton N, Breslin NP, Harman I, Bountra C, McLaren A, O'Morain CA. Increased mast cells in the irritable bowel syndrome. *Neurogastroenterol Motil* 2000; **12**: 449-457
30 **Chadwick VS**, Chen W, Shu D, Paulus B, Bethwaite P, Tie A, Wilson I. Activation of the mucosal immune system in irritable bowel syndrome. *Gastroenterology* 2002; **122**: 1778-1783
31 **Barbara G**, Vallance BA, Collins SM. Persistent intestinal neuromuscular dysfunction after acute nematode infection in mice. *Gastroenterology* 1997; **113**: 1224-1232

32 **Der T**, Bercik P, Donnelly G, Jackson T, Berezin I, Collins SM, Huizinga JD. Interstitial cells of cajal and inflammation-induced motor dysfunction in the mouse small intestine. *Gastroenterology* 2000; **119**: 1590-1599

33 **Bercík P**, Wang L, Verdú EF, Mao YK, Blennerhassett P, Khan WI, Kean I, Tougas G, Collins SM. Visceral hyperalgesia and intestinal dysmotility in a mouse model of postinfective gut dysfunction. *Gastroenterology* 2004; **127**: 179-187

34 **Spiller RC**. Postinfectious irritable bowel syndrome. *Gastroenterology* 2003; **124**: 1662-1671

35 **Sninsky CA**, Ramphal R, Gaskins DJ, Goldberg DA, Mathias JR. Alterations of myoelectric activity associated with Campylobacter jejuni and its cell-free filtrate in the small intestine of rabbits. *Gastroenterology* 1985; **89**: 337-344

36 **Lu G**, Qian X, Berezin I, Telford GL, Huizinga JD, Sarna SK. Inflammation modulates in vitro colonic myoelectric and contractile activity and interstitial cells of Cajal. *Am J Physiol* 1997; **273**: G1233-G1245

37 **Faussone-Pellegrini MS**, Gay J, Vannucchi MG, Corsani L, Fioramonti J. Alterations of neurokinin receptors and interstitial cells of Cajal during and after jejunal inflammation induced by Nippostrongylus brasiliensis in the rat. *Neurogastroenterol Motil* 2002; **14**: 83-95

38 **Chang IY**, Glasgow NJ, Takayama I, Horiguchi K, Sanders KM, Ward SM. Loss of interstitial cells of Cajal and development of electrical dysfunction in murine small bowel obstruction. *J Physiol* 2001; **536**: 555-568

39 **Spencer NJ**, Sanders KM, Smith TK. Migrating motor complexes do not require electrical slow waves in the mouse small intestine. *J Physiol* 2003; **553**: 881-893

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BRIEF ARTICLE

Is chronic hepatitis C virus infection a risk factor for breast cancer?

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Abstract

AIM: To evaluate the prevalence of breast tumors in adult females with chronic hepatitis C virus (HCV) infection.

METHODS: Prospective, single-center study, based on female outpatients consulting in a liver unit, for 1 year. The study group included females with present and/or past history of chronic infection by HCV. Patients with spontaneous recovery were excluded. Chronic hepatitis had been proved by liver biopsy in the majority of cases and/or biological markers of inflammation and fibrosis. The control group included female patients with other well documented chronic liver diseases: chronic hepatitis B, alcoholic liver disease, autoimmune hepatitis, hemochromatosis, non alcoholic liver disease, chronic cholangitis. Participating patients were prospectively questioned during consultation about past breast history and follow-up by mammography.

RESULTS: Breast carcinoma was recorded in 17/294 patients with HCV infection (5.8%, 95% CI: 3.1-8.4) *vs* 5/107 control patients (4.7%, 95% CI: 0.67-8.67). Benign tumors of the breast (mastosis, nodules, cysts) were recorded in 75/294 patients with HCV infection (25.5%, 95% CI: 20.5-30.5) *vs* 21/107 (19.6%, 95% CI: 12.1-27.1) in the control group. No lesion was noted in 202 patients with HCV (68.7%, 95% CI: 63.4-74) *vs* 81 control patients (75.7%, 95% CI: 67.6-83.8). Despite a trend to an increased prevalence in the group with HCV infection, the difference was not significant compared to the control group (P = NS). In patients over 40 years, the results were, respectively, as follows: breast cancer associated with HCV: 17/266 patients (6.3%, 95% CI: 3.4-9.3) *vs* 5/95 patients (5.2%, 95% CI: 0.7-9.7) in the control group; benign breast tumors: 72/266 patients with HCV infection (27%, 95% CI: 21.7-32.4) *vs* 18/95 patients (18.9%, 95% CI: 11-26.8) in the control group; no breast lesion 177/266 (66.5%, 95% CI: 60.9-72.2) in patients with HCV infection *vs* 72/95 (75.7%, 95% CI: 67.1-84.4) in the control group. The differences were not significant (P = NS).

CONCLUSION: These results suggest that chronic HCV infection is not a strong promoter of breast carcinoma in adult females of any age.

Key words: Breast tumors; Breast cancer; Hepatitis C virus infection; Risk factor

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INTRODUCTION

Several viruses have been involved in the occurrence of cancers[1]. For instance, human papilloma virus has been directly implicated in uterus cancer; poliomavirus in mesothelioma and brain tumors; Epstein-Barr virus in B-cell lymphoproliferative disease and nasopharyngeal carcinoma; herpes virus in Kaposi sarcoma[1]. Hepatitis C virus (HCV) is well-known to cause chronic hepatitis, cirrhosis and hepatocarcinoma[2-5]. The prevalence of HCV in France, as in other Western European countries, is around 1% and is estimated to be 1.6% in the United States[6-8]. The potential link between HCV infection and the risk of developing malignancy other than hepatocarcinoma has been recently raised in several studies[9-13]. There are several lines of evidence showing a role in the occurrence in non-Hodgkin lymphoma and lymphoproliferative diseases[9,10]. Recent studies argue for an increasing risk of intra-hepatic cholangiocarcinoma[11] and thyroid cancer[12]. The prevalence of HCV has been evaluated in elderly patients with tumors different from hepatocarcinoma and non-Hodgkin lymphoma (colorectal, prostate, breast, bladder, kidney)[13]. Among 236 patients, 87 (36%) were positive for HCV, a higher prevalence than in the patients of the control group (10%)[13]. A statistically significant difference was observed with kidney cancer, prostate cancer, and bladder cancer[13]. Finally, the link between hepatocarcinoma and another tumor has been assessed in a retrospective study including 37 patients[14]. Five patients (13.5%) had developed another primary cancer before or after hepatocarcinoma: kidney cancer, breast cancer, colorectal cancer, prostate cancer, or lymphoma. A common point between these 5 patients was HCV chronic infection. This suggested that HCV chronic infection may not only promote hepatocarcinoma, but also other solid tumors[14].

Therefore the aim of this study was to assess the frequency of breast tumors in adult females with chronic infection by HCV and whether this disease may be a promoting factor for the onset of benign or malignant breast tumors.

MATERIALS AND METHODS

This was a prospective, single-center study performed over 1 year in female patient aged ⩾ 20 years, consulting in the Liver Unit of Montpellier School of Medicine, France, for chronic liver diseases. The study group included patients with present or past chronic infection by HCV.

Patients

Inclusions criteria: Age ⩾ 20 years, the evidence of chronic infection by HCV based on the presence of serum anti-HCV antibodies, detection of serum HCV RNA by PCR tested on several occasions for a period longer than 1 year; chronic hepatitis C proved by liver biopsy (75% of patients) or non-invasive methods (25% of patients) including biological markers of inflammation and fibrosis of the liver such as Fibrotest-Actitest® and/or elastographic examination (Fibroscan®) as recently published[15-17]; agreement of the patient for participation in the study.

Exclusion criteria: A spontaneous recovery from HCV; co-infection by hepatitis B virus or human immunodeficiency virus; absence of capacity to understand or to answer the questions in the inquiry.

The control group included females seen sequentially and prospectively during the same period and affected by chronic liver disease over 1 year, with well defined characteristics based on clinical, radiological and histological features [chronic hepatitis B, chronic alcoholic liver disease, auto-immune hepatitis, hemochromatosis, non alcoholic fatty liver disease (NAFLD), chronic cholangitis, *etc.*].

Methods

The following information was collected during the consultation by using a questionnaire: past history of breast cancer or benign breast tumor; which type if any (adenoma, mastosis, cyst); performed examinations or treatment (mammography, biopsy, surgery); potential participation in a tumor detection program by mammography. Indeed, in our geographic area, there is a detection program for breast lesions with systematic mammography every 2 years for females over 40 years.

Statistical analysis

The data processing was performed using SAS software packages version 8.1. A general descriptive analysis was done for every parameter of the study. The distribution of qualitative variables (such as breast tumors) between groups was compared using χ^2 test. When the calculated frequency of the categorical data of the contingency table did not allow the use of the χ^2 test, Fisher's exact test was performed. A P level < 0.05 was considered as significant. Stratification was performed according to age brackets: 20 to 40 years, 41 to 60 years and more than 60 years. Unilateral and bilateral power was calculated *a posteriori*.

RESULTS

Four hundred and one patients fulfilled inclusion criteria and all agreed to participate in the study. The study group with HCV infection included 294 patients; the control group included 107 patients with the following chronic liver diseases: NAFLD, 32 cases; chronic hepatitis B, 10 cases; primary biliary cirrhosis, 17 cases; auto-immune chronic hepatitis, 13 cases; chronic alcoholic liver disease, 4 cases; chronic cholangitis, 9 cases; hemochromatosis, 4 cases; and other chronic liver diseases, 18 cases.

Patients' ages ranged from 21 to 84 years (median 58 years) in the HCV group and from 23 to 84 years (median 56 years) in the control group. The distribution by age was comparable in the two groups with a predominance of patients between 40 and 70 years: 20-40 years, n = 36 (8.9%) *vs* n =

Table 1 Characteristics of the 294 patients with chronic hepatitis C

	All patients
Median age (yr)	58 (21-84)
HCV Genotype	
HCV 1	63%
HCV 2	11%
HCV 3	19%
HCV 4-5	7%
Severity of liver disease[1]	
F0-F2	70%
F3	16%
F4	14%
HCV treatment	
Never treated	30%
Past or ongoing treatment	70%

[1]The extent of fibrosis has been expressed according to the METAVIR scale as previously described[15]. HCV: Hepatitis C virus.

15 (9.7%); 41-60 years, n = 132 (33%) *vs* n = 52 (45.9%); > 60 years, n = 126 (42.8%) *vs* n = 40 (37.4%). Other main characteristics of patients with chronic HCV infection, including genotype, severity of fibrosis and anti-viral treatment history, are shown in Table 1. They are similar to the features collected in the data bank of patients with HCV infection in our regional HCV network (3280 patients)[18]. In the control group, the percentage of chronic liver diseases reaching the stage of cirrhosis (stage F4 of METAVIR scale) was 13% (14/107 cases), which was similar to the HCV group (14%) (Table 1).

The programme of mammography for patient aged over 40 years was followed in 212/266 patients (79.7%) of the HCV group and in 74/99 patients (77.8%) of the control group. In younger patients, mammography had only been performed because of symptoms, in less than 15% of patients of both groups.

The prevalence of breast tumors is shown in Table 2. Among all patients, breast cancer was recorded in 5.8% (95% CI: 3.1-8.4) of HCV group patients *vs* 4.7% (95% CI: 0.67-8.67), a benign breast tumor in 25.5% (95% CI: 20.5-30.5) in the HCV group *vs* 19.6% (95% CI: 12.1-27.1) in the control group, no evidence of breast lesion in 68.7% (95% CI: 63.4-74) of patients in the HCV group *vs* 75.7% (95% CI: 67.6-83.8) in the control group. Despite a trend for a higher prevalence of malignant or benign tumors in the HCV group, there was no significant statistical difference with the control group (Table 2). Familial history of breast cancer was recorded only in 1 of the 17 patients in the HCV group and none in the 5 cases of the control group.

The same analysis was performed according age brackets as presented in Table 3. No breast cancer was recorded in females younger than 40 years in the two groups. The frequency was low for females between 41 and 60 years, with a mild predominance but no significant difference in the HCV group compared to the control group: 3.4% (95% CI: 0.5-6.4) *vs* 1.8% (95% CI: 0-5.3). Females older than 60 years exhibited the highest prevalence with 10.0% without any difference between the two groups. In all patients over 40 years, breast cancer in the HCV group was 17/266 patients (6.3%, 95% CI: 3.4-9.3) *vs* 5/95 (5.2%, 95% CI: 0.7-9.7) in the control group.

For benign breast tumors, frequency also varied according to age brackets: it was slightly lower in the HCV group *vs* the control group for females between 20 and 40 years. In contrast, it was slightly higher in the other two age brackets but the difference was not statistically significant.

The absence of breast tumors was slightly higher in females aged between 41 and 60 years, and older than 60 years in the control group *vs* the HCV group but the difference was not statistically significant.

DISCUSSION

In many parts of the world, breast cancer is the most frequent form of cancer in females[19-22]. Similarly in France, there are 49 000 new cases/year and 11 000 deaths for a population of 60 million inhabitants[23-25]. The incidence is 101 cases/100 000 females[25]. Overall, cancer occurs in one female out of 11. As with many other cancers, the risk increases with age (less than 10% of breast cancers are detected in patients younger than 40 years[21-24]. Then the incidence increases with age[21-24]. These observations justify a systematic detection in females from 50 years. The causes of breast cancer are poorly known. Nevertheless, some risk factors have been identified[25-28]: benign breast diseases, fertility (females without pregnancy or with first pregnancy later than 30 years old exhibit a higher risk), obesity particularly after menopause[25]. Familial and genetic factors may also increase the risk, in particular through a gene mutation (BRCA1-BRCA2)[28]. The role of oral contraceptives has been discussed[21-27]. The increase in risk has been mainly observed in oral contraceptive users with a family history of breast cancer[28].

The role of HCV in breast cancer has been recently raised[14] for the following reasons. Chronic HCV infection is clearly involved in the occurrence of hepatocarcinoma and lymphoma[3,4-10] and in several other solid tumors[11-13]. Some cases of breast cancer were observed in a recent study of patients with HCV[14] and several cases have been recorded during the regular follow-up of the large cohort of patients with chronic HCV infection seen in the Liver Unit of Montpellier School of Medicine (personal observation). This led to the present prospective study knowing that a program of systematic detection of breast tumors by mammography every 2 years in all females older than 40 years has been in place in our geographic area for nearly 20 years.

Global results of this study show a breast cancer frequency of 5.8% in adult females with chronic HCV infection. Intentionally, we included a group of young females, aged 20-40 years, to detect a potential signal in an age population known to not exhibit a particular risk. No malignant lesion was recorded. However, only a small proportion of these patients had undergone mammography. Therefore, the detection of a tumor was mainly based on

Table 2 Prevalence of breast tumors

	Patients with HCV infection (n = 294)		Patients with other chronic liver diseases (n = 107)		P value
	n (%)	95% CI	n (%)	95% CI	
Breast cancer - all patients	17 (5.8)	3.1-8.4	5 (4.7)	0.67-8.67	NS
Benign breast tumors	75 (25.5)	20.5-30.5	21 (19.6)	12.1-27.1	NS
No breast lesion	202 (68.7)	63.4-74	81 (75.7)	67.6-83.8	NS

HCV: Hepatitis C virus; NS: Not significant.

Table 3 Prevalence of breast tumors according to age

Age of patients (yr)	Patients with chronic HCV infection (n = 294)		Patients with other chronic liver diseases (n = 107)		P value
	n (%)	95% CI	n (%)	95% CI	
Breast cancer					
20-40	0/28 (0)		0/12 (0)		NS
41-60	5/146 (3.4)	0.5-6.4	1/55 (1.8)	0-5.3	NS
> 60	12/120 (10.0)	4.6-15.4	4/40 (10.0)	0.7-19.3	NS
Benign breast tumors					
20-40	3/28 (10.7)	0-22.2	3/12 (25.0)	0.5-49.5	NS
41-60	41/146 (28.1)	20.8-35.3	12/55 (21.8)	10.9-32.7	NS
> 60	31/120 (25.8)	18-33.7	6/40 (15.0)	3.9-26.1	NS
No breast lesion					
20-40	25/28 (89.3)	77.8-100	9/12 (75.0)	50.5-99.5	NS
40-60	100/146 (68.5)	60.9-76	42/55 (76.4)	65.1-87.6	NS
> 60	77/120 (64.2)	55.6-72.7	30/40 (75.0)	61.6-88.4	NS

HCV: Hepatitis C virus; NS: Not significant.

its clinical expression. This sub-group representing about 10% of the overall group has slightly lowered the global prevalence. Indeed, the prevalence of all patients aged more than 40 years is 6.3% and reaches its highest rate, 10%, in females aged more than 60 years. The prevalence may have been underestimated since the mammography program was not followed in 20% of patients. Results observed in the HCV group were similar to those found in the control group, including females with other types of chronic liver diseases and having the same breast tumor detection program. We observed a similar low frequency in younger patients and the same proportion of patients who did not follow the mammography program. Therefore, this did not influence the comparison between groups. Finally, a familial history of breast cancer was recorded in a single patient with breast cancer (in the HCV group). This factor does not appear to have influenced the result of our study. Overall, these data do not support the idea that HCV chronic infection is a factor which contributes markedly to breast cancer. This view is also reinforced by the fact that prevalence of breast cancer found in this study is within the range of those found in general French and occidental populations[19-24]. Nevertheless, the interpretation of the results needs to be balanced by some limitations, in particular the relatively low number of patients in the control group and the absence of a priori calculation of the number of patients required to show a significant difference between groups with high power. This is largely caused by the fact that the prevalence of breast cancer in patients with chronic liver diseases in general and in HCV infection in particular, was completely unknown when the study was started. Therefore, our study does not allow us to draw definite conclusions. Nevertheless, it may serve as basis to set a more powerful study with matched control groups.

In summary, results of this study allowed the evaluation of the prevalence of breast cancer in patients with HCV chronic infection and suggest that HCV is not a strong promoter of breast carcinoma in adult females of any age.

COMMENTS

Background

Chronic infection by hepatitis C virus (HCV) exhibits a high frequency worldwide and represents a major cause of chronic liver disease leading to cirrhosis and hepatocarcinoma. Its influence on the onset of malignant tumors is under investigation.

Research frontiers

Several recent studies suggest that HCV chronic infection can not only cause hepatocarcinoma and lymphoma but may also promote the onset of several other solid tumors involving biliary ducts, thyroid, prostate, kidney and bladder.

Innovations and breakthroughs

The prevalence of breast cancer in patients with chronic liver diseases in general and HCV chronic infection in particular, is unknown. The relationship between HCV infection and breast cancer has been recently suggested by anecdotal cases. This is the first study designed to evaluate the prevalence of breast malignant and benign tumors in female patients and to assess whether HCV chronic infection is a risk factor. The study has been performed prospectively, using other chronic liver diseases as the control group. The results show the same prevalence of breast tumors in both groups which suggests that HCV does not appear as a strong promoting factor.

Applications

This study has allowed us to estimate the prevalence of breast cancer in females with chronic HCV infection. The interpretation of the results is balanced

by the number of patients included in the study and statistical power. Nevertheless, this constitutes a step to design new studies with matched control groups including a much larger number of patients to evaluate a potential low impact of HCV in breast malignancy.

Peer review

The authors evaluated the association between HCV infection and breast cancer. The study included 294 patients with HCV infection. Control subjects were 107 women seen in the same liver clinic over a 1-year period. Overall, there was no difference in the frequency of breast cancer or benign breast lesions between HCV-infected patients and control subjects. The hypothesis is interesting, but the study has limitations as discussed.

REFERENCES

1 **Pagano JS**, Blaser M, Buendia MA, Damania B, Khalili K, Raab-Traub N, Roizman B. Infectious agents and cancer: criteria for a causal relation. *Semin Cancer Biol* 2004; **14**: 453-471
2 **Ghany MG**, Strader DB, Thomas DL, Seeff LB. Diagnosis, management, and treatment of hepatitis C: an update. *Hepatology* 2009; **49**: 1335-1374
3 **Sherman M**. Hepatocellular carcinoma: epidemiology, risk factors, and screening. *Semin Liver Dis* 2005; **25**: 143-154
4 **Degos F**, Christidis C, Ganne-Carrie N, Farmachidi JP, Degott C, Guettier C, Trinchet JC, Beaugrand M, Chevret S. Hepatitis C virus related cirrhosis: time to occurrence of hepatocellular carcinoma and death. *Gut* 2000; **47**: 131-136
5 **Hassan MM**, Frome A, Patt YZ, El-Serag HB. Rising prevalence of hepatitis C virus infection among patients recently diagnosed with hepatocellular carcinoma in the United States. *J Clin Gastroenterol* 2002; **35**: 266-269
6 **Martinot-Peignoux M**, Roudot-Thoraval F, Mendel I, Coste J, Izopet J, Duverlie G, Payan C, Pawlotsky JM, Defer C, Bogard M, Gerolami V, Halfon P, Buisson Y, Fouqueray B, Loiseau P, Lamoril J, Lefrere JJ, Marcellin P. Hepatitis C virus genotypes in France: relationship with epidemiology, pathogenicity and response to interferon therapy. The GEMHEP. *J Viral Hepat* 1999; **6**: 435-443
7 **Wasley A**, Alter MJ. Epidemiology of hepatitis C: geographic differences and temporal trends. *Semin Liver Dis* 2000; **20**: 1-16
8 **Armstrong GL**, Wasley A, Simard EP, McQuillan GM, Kuhnert WL, Alter MJ. The prevalence of hepatitis C virus infection in the United States, 1999 through 2002. *Ann Intern Med* 2006; **144**: 705-714
9 **Schöllkopf C**, Smedby KE, Hjalgrim H, Rostgaard K, Panum I, Vinner L, Chang ET, Glimelius B, Porwit A, Sundström C, Hansen M, Adami HO, Melbye M. Hepatitis C infection and risk of malignant lymphoma. *Int J Cancer* 2008; **122**: 1885-1890
10 **Giordano TP**, Henderson L, Landgren O, Chiao EY, Kramer JR, El-Serag H, Engels EA. Risk of non-Hodgkin lymphoma and lymphoproliferative precursor diseases in US veterans with hepatitis C virus. *JAMA* 2007; **297**: 2010-2017
11 **El-Serag HB**, Engels EA, Landgren O, Chiao E, Henderson L, Amaratunge HC, Giordano TP. Risk of hepatobiliary and pancreatic cancers after hepatitis C virus infection: A population-based study of U.S. veterans. *Hepatology* 2009; **49**: 116-123
12 **Montella M**, Pezzullo L, Crispo A, Izzo F, Amore A, Marone U, Tamburini M, Ronga D, Chiofalo MG, Chiappetta G, Mozzillo N. Risk of thyroid cancer and high prevalence of hepatitis C virus. *Oncol Rep* 2003; **10**: 133-136
13 **Malaguarnera M**, Gargante MP, Risino C, Ranno S, Berretta M, Cannizzaro MA, Costanzo M, Fricia T, Rampello E, Romano M. Hepatitis C virus in elderly cancer patients. *Eur J Intern Med* 2006; **17**: 325-329
14 **Bruno G**, Andreozzi P, Graf U, Santangelo G. Hepatitis C virus: a high risk factor for a second primary malignancy besides hepatocellular carcinoma. Fact or fiction? *Clin Ter* 1999; **150**: 413-418
15 **Bedossa P**, Poynard T. An algorithm for the grading of activity in chronic hepatitis C. The METAVIR Cooperative Study Group. *Hepatology* 1996; **24**: 289-293
16 **Imbert-Bismut F**, Ratziu V, Pieroni L, Charlotte F, Benhamou Y, Poynard T. Biochemical markers of liver fibrosis in patients with hepatitis C virus infection: a prospective study. *Lancet* 2001; **357**: 1069-1075
17 **Castera L**, Denis J, Babany G, Roudot-Thoraval F. Evolving practices of non-invasive markers of liver fibrosis in patients with chronic hepatitis C in France: time for new guidelines? *J Hepatol* 2007; **46**: 528-529; author reply 529-530
18 **Bakanova A**, Mura T, Mourrut C, Blanc P, Pageaux GP, Remy AJ, Chiari R, Larrey D, Fabbro-Peray P, Ribard D. Le dossier informatisé des patients atteints d'hépatite C en Languedoc-Roussillon: un bilan après 29 mois de fonctionnement. *Gastroenterol Clin Biol* 2007; **31**: A140
19 **Rochefort H**, Rouëssé J. [How to reduce the incidence of breast cancer] *Bull Acad Natl Med* 2008; **192**: 161-179
20 **Kelsey JL**, Gammon MD. The epidemiology of breast cancer. *CA Cancer J Clin* 1991; **41**: 146-165
21 **Piscitelli P**, Santoriello A, Buonaguro FM, Di Maio M, Iolascon G, Gimigliano F, Marinelli A, Distante A, Serravezza G, Sordi E, Cagossi K, Artioli F, Santangelo M, Fucito A, Gimigliano R, Brandi ML, Crespi M, Giordano A. Incidence of breast cancer in Italy: mastectomies and quadrantectomies performed between 2000 and 2005. *J Exp Clin Cancer Res* 2009; **28**: 86
22 **Chia S**, Bryce C, Gelmon K. The 2000 EBCTCG overview: a widening gap. *Lancet* 2005; **365**: 1665-1666
23 **Tavani A**, Gallus S, La Vecchia C, Negri E, Montella M, Dal Maso L, Franceschi S. Risk factors for breast cancer in women under 40 years. *Eur J Cancer* 1999; **35**: 1361-1367
24 **Peppercorn J**. Breast cancer in women under 40 years. *Oncology* 2009; **23**: 465-474
25 **Lajous M**, Boutron-Ruault MC, Fabre A, Clavel-Chapelon F, Romieu I. Carbohydrate intake, glycemic index, glycemic load, and risk of postmenopausal breast cancer in a prospective study of French women. *Am J Clin Nutr* 2008; **87**: 1384-1391
26 **Terry KL**, Willett WC, Rich-Edwards JW, Michels KB. A prospective study of infertility due to ovulatory disorders, ovulation induction, and incidence of breast cancer. *Arch Intern Med* 2006; **166**: 2484-2489
27 **Casey PM**, Cerhan JR, Pruthi S. Oral contraceptive use and risk of breast cancer. *Mayo Clin Proc* 2008; **83**: 86-90; quiz 90-91
28 **Hamilton R**, Williams JK, Bowers BJ, Calzone K. Life trajectories, genetic testing, and risk reduction decisions in 18-39 year old women at risk for hereditary breast and ovarian cancer. *J Genet Couns* 2009; **18**: 147-159

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BRIEF ARTICLE

Nasogastric or nasointestinal feeding in severe acute pancreatitis

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Abstract

AIM: To assess the rate of spontaneous tube migration and to compare the effects of naso-gastric and naso-intestinal (NI) (beyond the ligament of Treitz) feeding in severe acute pancreatitis (SAP).

METHODS: After bedside intragastric insertion, tube position was assessed, and enteral nutrition (EN) started at day 4, irrespective of tube localization. Patients were monitored daily and clinical and laboratory parameters evaluated to compare the outcome of patients with nasogastric (NG) or NI tube.

RESULTS: Spontaneous tube migration to a NI site occurred in 10/25 (40%) prospectively enrolled SAP patients, while in 15 (60%) nutrition was started with a NG tube. Groups were similar for demographics and pancreatitis aetiology but computed tomography (CT) severity index was higher in NG tube patients than in NI (mean 6.2 *vs* 4.7, P = 0.04). The CT index seemed a risk factor for failed obtainment of spontaneous distal migration. EN trough NG or NI tube were similar in terms of tolerability, safety, clinical goals, complications and hospital stay.

CONCLUSION: Spontaneous distal tube migration is successful in 40% of SAP patients, with higher CT severity index predicting intragastric retention; in such cases EN by NG tubes seems to provide a pragmatic alternative opportunity with similar outcomes.

Key words: Acute pancreatitis; Enteral feeding; Tube migration; Nasogastric; Safety

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INTRODUCTION

Acute pancreatitis (AP) is an acute inflammatory process which has different grades of severity and is characterized by high mortality rates in the case of infected pancreatic necrosis[1].

Severe AP (SAP) with pancreatic necrosis is therefore a hard challenge for clinicians, and its management is still debated. The aim of treatment is to prevent necrosis

infection and to manage the hypercatabolism secondary to extended pancreatic and extrapancreatic inflammation with an adequate nutritional, volumetric and hydroelectrolytic support. Nutritional support is presently considered a key issue in patient management. Enteral nutrition (EN) should be preferred to total parenteral nutrition (TPN) in patients with SAP[2,3], as also suggested by current guidelines[1,4-6]. EN is indeed associated with reduced mortality, lower septic complications, reduced surgical procedures and hospital stay[2,3], possibly owing to a trophic action on the intestinal wall and prevention/reduction of bacterial translocation.

However, in clinical practice EN is employed far less frequently than it should. A recent survey conducted by the Italian Society of Pancreatology has reported that only about 20% of SAP patients received EN, either as single nutritional support, or in combination with TPN[7]. This figure is slightly more encouraging in Holland where some 50% of patients with SAP received EN in an observational multicentre study[8].

The main obstacle to EN diffusion is that it is considered complicated, and to require specific skills. Indeed, to ensure full pancreatic rest, nutrition tubes should be placed in the jejunum[9,10]. Although spontaneous transpyloric migration of tubes after gastric positioning, and subsequent localization in the distal duodenum or jejunum, is possible, few studies have specifically addressed this issue in patients with AP. Endoscopic placement of a nasojejunal tube is a possible alternative, but it is troublesome, potentially risky, and variable success rates have been reported[11]. Other techniques and devices have been proposed to improve tube positioning beyond the ligament of Treitz[12-14], but results, although appealing, are preliminary and sometimes out of reach in daily clinical practice.

In the past few years, it has been proposed that EN through nasogastric (NG) tubes may be a simple, safe and equally valid alternative to nasojejunal tubes, with the potential advantage of earlier administration of nutrients[15-17]. However, NG feeding cannot be recommended at this time, and it is not clear if a subgroup of SAP patients may benefit more from this approach.

We speculated that a pragmatic possibility in real-world clinical practice would be to employ NG feeding whenever tube migration does not occur spontaneously.

The aims of this study were therefore to assess the rate of spontaneous distal migration of EN tubes in patients with predicted SAP, to identify possible factors associated with it, and to compare the safety and tolerability of EN with an elemental formula in patients who started nutrition with a "proximal", NG or a "distal", naso-intestinal (NI) tube, depending on the success of spontaneous tube migration.

MATERIALS AND METHODS

Patients

This is a pragmatic ("real world") study, prospectively evaluating patients with predicted SAP admitted to our Institution from January 2006 to November 2009. AP was defined by the presence of typical abdominal pain associated with serum amylase levels > 3 times normal value. Patients with predicted SAP, as defined by a Ranson's score of 3 or higher and/or by a CT severity index of 4 or higher (as reviewed in 1), were included.

Treatment protocol

SAP patients received appropriate fluid support, antibiotic prophylaxis with iv imipenem (500 mg every 8 h), antisecretory therapy with iv pantoprazole (40 mg once daily), and were offered EN. The EN protocol included positioning of a feeding tube (10 F Flexiflo tungsten-weighted polyurethane feeding tube**,** Abbott, Baltimore, USA). After lubrication with 20 mL of water, intubation was performed at the bedside in all cases and, once completed and verified by X-ray, the guide-wire was withdrawn and the tube fixed to the nose. Prokinetics (metoclopramide 10 mg) were administered twice a day for 3 d after intubation. Tip position was radiologically assessed after 24 (day 1) and 72 h (day 3). Position was considered "proximal" (NG) if the tube tip was in the stomach or in the duodenum, proximal to the ligament of Treitz, and "distal" (NI) if in the duodenum beyond the ligament of Treitz or in the jejunum. At day 4 EN was started irrespectively of tube position (either "NG" or "NI"). An elemental formula (Survimed®) was employed at increasing volumes, from 20 mL/h up to an energetic target of 2000 kcal per day (100 mL/h).

Measured outcomes

Patients were monitored daily by measurement of clinical and laboratory parameters, and pain through a quantitative score, based on the subjective evaluation and the need for analgesic drugs (0 = no pain, 1 = pain with no need of analgesics, 2 = pain responding to low dose analgesics, 3 = pain responding to high dose analgesics, 4 = pain not responding to high dose analgesics).

Pain recurrence, biochemical changes (amylase, lipase and C-reactive protein), side effects (such as nausea, vomiting or diarrhoea), success of EN in terms of caloric target and days necessary to reach it, as well as possible need to TPN switching, were recorded. Patients received further appropriate clinical and radiologic investigations when needed. Occurrence of pancreatic (infected necrosis) and/or extrapancreatic complications (renal and/or respiratory failure, bleeding) were also recorded, as well as the patients' clinical outcome (mortality, need for surgery) and length of hospital stay.

Statistical analysis

Categorical data (percentages) were compared by means of Fisher's exact test, and continuous data by means of *t*-test for independent samples. Possible associated risks were evaluated by logistic regression analysis.

RESULTS

Patients

During the study protocol, 116 patients with AP were admitted to our unit. Their demographics and clinical features are detailed in Table 1. Among them, there were 28 pa-

Table 1 Characteristics of the 116 patients with acute pancreatitis hospitalized in the study period *n* (%)

Gender (female/male)	53/63
Median age (range, yr)	55.5 (17-92)
Mild/severe pancreatitis	88/28
Etiology	
Biliary	59 (50.9)
Alcoholic	28 (24.1)
Drug-induced	4 (3.4)
Idiopathic	8 (6.9)
Hypertrigliceridemia	3 (2.6)
Iatrogenic	6 (5.2)
Autoimmune	1 (0.9)
Traumatic	1 (0.9)
Pancreas divisum	4 (3.4)
Intrapapillary mucinous tumour	2 (1.7)

Table 2 Demographics and clinical features of the 25 patients with predicted severe acute pancreatitis who received enteral nutrition, according to tube position at nutrition start

	Nasogastric (*n* = 15)	Nasointestinal (*n* = 10)	*P*
Sex (female, %)	6 (40)	4 (40)	1
Median age (yr)	56 (31-83)	63 (36-89)	0.3
BMI (kg/m^2)	24.8 (21.4-28.2)	25.4 (21.8-29.1)	0.7
Amylase at entry (U/L; nv < 110)	1045.5 (592.2-1498.8)	1141.6 (127.7-2155.4)	0.8
Lipase at entry (U/L; nv < 300)	8559.8 (3676.4-13443.2)	14037 (2026.1-30100.1)	0.4
CT severity index	6.2 (5.1-7.2)	4.7 (3.5-5.8)	0.04
Ranson's score	3.8 (3.1-4.6)	3 (1.6-4.3)	0.2
CRP at 72 h (mg/L)	149.1 (82.5-215.6)	138 (27-249)	0.8
White blood dells count at entry	13620 (10476-16760)	9940 (5200-14675)	0.1
LDH at entry (mU/mL)	841.8 (608.8-1074.7)	862.6 (244.8-1480.4)	0.9
Hematocrit at entry (%)	38.6 (33.9-43.2)	39.6 (35.9-43.2)	0.7
Etiology: biliary/ alcoholic or other	6/9	5/5	0.6

Values expressed as total number (percentage) or as mean (95% CI), but for age. BMI: Body mass index; CT: Computerized tomography; CRP: C reactive protein; LDH: Lactate dehydrogenase.

tients with predicted SAP (24.1%) who were offered EN as part of their treatment. Two patients refused tube positioning and received TPN, another patient spontaneously withdrew the tube on day 1 and refused further invasive treatments. Data concerning the remaining 25 patients were analysed.

Rate of spontaneous nutrition tube migration

Plain abdominal X-ray evaluation at day 3 demonstrated successful transpyloric tube migration and "NI" positioning in 10 (40%) patients. The tube did not migrate and remained "NG" in the other 15 patients (60%). As shown in Table 2, the two groups were similar in terms of sex, age and pancreatitis etiology. The predicted severity was not different according to Ranson's score, C-reactive protein or other biochemical values, but the CT severity index in the NG tube group was significantly higher than the NI group (mean 6.2 *vs* 4.7, $P = 0.04$). At a logistic regression analysis, we could not identify factors associated with the NG tube position, although CT severity index was the variable closest to significance (OR = 1.6 per unit, 95% CI: 0.95-2.9). Moreover, in all 4 patients (100%) with a CT severity index > 6, the tube did not migrate beyond the pylorus, compared to 11 of the 21 patients (52.3%) with an index ⩽ 6; however this difference was not statistically significant, probably due to the small number of patients.

Table 3 Tolerability and success of nutrition according to tube position *n* (%)

	Nasogastric (*n* = 15)	Nasointestinal (*n* = 10)	*P*
Tube malpositioning	0	0	-
Epistaxis or Sinusitis	1 (6.6)	1 (10)	1
Accidental tube removal	0	1 (10)	0.4
Tube clogging	1 (6.6)	0	1
Aspiration pneumonia	0	0	-
Exacerbation of pain	5 (33.3)	2 (20)	0.68
Vomiting	2 (13.3)	1 (10)	1
Diarrhoea	5 (33.3)	3 (30)	1
Amylase increase > 10%	0	0	-
Lipase increase > 10%	1 (6.6)	0	1
CRP increase > 10%	2 (13.3)	2 (20)	1
Need to switch to TPN	4 (26.6)	0	0.27
Energetic target reached	14 (93.3)	8 (80)	1
Days to caloric target, mean (95% CI)	5.6 (3.8-7.4)	4.3 (3.1-5.6)	0.3

TPN: Total parenteral nutrition.

Safety and tolerability of nutrition

There were no differences regarding complications of the feeding tube positioning, such as malpositioning, epistaxis or aspiration pneumonia between patients with a NG or a NI tube. Moreover, after EN start, there was no significant difference between the NG and the NI tube groups in terms of exacerbation of pain, biochemical changes (amylase, lipase and C-reactive protein), side effects or need to switch to TPN. A similar high percentage of patients reached the energetic target (2000 Kcal) in both groups without significant time difference (Table 3).

Clinical outcome

As detailed in Table 4, there was no significant difference in the clinical outcome between the two groups, although more complications occurred in the NG group.

DISCUSSION

In the present study spontaneous migration of the EN tube beyond the stomach occurred in 40% of predicted SAP patients, and a higher CT severity index was associated with the tube being retained in the stomach. However, EN was successfully delivered in some 90% of cases, even in those patients in which tube migration beyond the ligament of Treitz was unsuccessful. Similar results in terms of safety and tolerability were observed in patients with

Table 4 Clinical outcomes of patients according to tube position *n* (%)

	Nasogastric (*n* = 15)	Nasointestinal (*n* = 10)	*P*
Mortality	0	0	-
Need of surgery	0	0	-
Complications			
Infected pancreatic necrosis	3 (20)	1 (10)	1
Renal failure	1 (6.6)	0	1
Respiratory failure	2 (13.3)	0	0.1
Bleeding	1 (6.6)	0	1
Any of the above complications	4 (26.6)	1 (10)	0.6
Total hospital stay, mean (95% CI)	30.6 (18.1-43)	21.2 (17.7-24.6)	0.1

an "NG" or an "NI" tube. Furthermore, both approaches were equally effective in providing the nutritional support needed, caloric goals were reached in similar time intervals and length of hospital stay was not different.

A first interesting result regards the rate of spontaneous tube migration after bedside positioning, without endoscopic or radiologic assistance. The feeding tube migrated to a NI position in 40% of cases, and patients with the NG tube had a significantly higher CT scan severity index. Bedside tube positioning caused only few mild complications without differences between the two groups, but no cases of aspiration pneumonia occurred. This finding is relevant, as although delivery of feeding into the small bowel should be associated with a lower risk of aspiration, there are few data supporting this view[18].

The rate of spontaneous distal tube migration with unguided probing is considered to be around 50% in patients admitted to intensive care units (ICU) for different diseases[19]. Few studies have reported these data specifically for patients with predicted SAP, with success rates of spontaneous migration ranging from 60% to 80%[20,21]. Our results are in agreement with a recent French study reporting an overall successful migration in 61% of patients with either mild or severe AP, with this rate being reduced to 48% in SAP patients having a CT severity index score $\geqslant 4$[21]. Similarly, in our experience, in all patients with extensive pancreatic necrosis (CT severity index > 6), the tube did not migrate beyond the pylorus, and the CT index seemed a risk factor associated with failed spontaneous migration. These data may be explained by an impaired transpyloric migration due to gastroparesis, or to mechanical obstacles caused by local oedematous reactions and/or fluid collections present in the most advanced SAP cases.

As far as tolerability and safety are concerned, we have not observed any significant difference between patients receiving EN either by NG or NI tube. The nutritional goal was reached in 93% of NG patients and 80% of NI. Our findings seem to be in agreement with those published by Eatock *et al*[15], who had randomized 49 SAP patients into two groups, administering EN through NG tube in 27 cases and through NI tube in the remaining 22. Patients had been monitored daily by severity index and pain score to evaluate changes in AP severity due to enteral feeding, and during hospital stay groups behaved similarly, no matter the kind of EN used. Another two randomized clinical trials[16,17] dealing with NG enteral feeding have supported the safety and efficacy of this nutritional route, and subsequent meta-analyses confirmed the lack of difference between the two approaches, although the paucity of available data was underlined as a factor limiting the evaluation[22,23].

Regarding clinical outcomes, we have not found any significant difference in terms of complications, mortality and length of hospital stay between the two groups, although most complications occurred in patients receiving NG feeding (Table 4). This small, not significant gap is probably due to a higher prevalence of extensive necrosis in the NG group, accordingly to the significant higher CT scores of these patients at entry. However, since our group of predicted SAP patients did not experience prolonged organ failure which is a key event in discriminating patients with more severe forms[24], and we observed absence of mortality in both groups, the findings obtained in our study may not apply to patients with SAP and prolonged multiple organ failure.

This is the first study of its kind observing the outcome of EN in SAP patients in a "real world" clinical setting, with the study protocol driven by the need to have more solid grounds in making clinical decisions about everyday medical care circumstances. Both the proximal and the distal enteral approaches result to be feasible, safe and effective in most patients.

The working hypothesis we wanted to test, and that seems to be confirmed by our results, was that when spontaneous tube migration fails EN can be safely administered through NG tube. This issue has a relevant impact on everyday clinical practice as the main limit to EN usage in AP is the technical difficulty in obtaining small bowel access, as reported by 72% of ICUs joining a national survey in Canada[25].

Of course, the present non-randomized study design cannot highlight the potential benefits of NG nutrition, such as the possibility of immediate start of EN after tube positioning, but only the potential harms caused by stimulation of pancreatic function. However our observation may support the need for further clinical research aimed at clarifying this issue. Furthermore, as the rate of spontaneous distal migration of the nutrition tube, and factors related with it, was one of the results the study was aimed at identifying, the design could not imply a randomization between NG and NI, nor a power calculation. As a consequence, it is possible that differences between groups have not been appreciated due to underpowered samples. In this view, the ongoing multicentre trial on gastric *vs* mid-jejunal feeding funded by the National Institutes of Health will probably provide further important information (http://clinicaltrials.gov Identifier: NCT00580749).

In conclusion, spontaneous distal tube migration in patients with predicted SAP is successful in 40% of patients, and CT severity index is higher in patients with failed distal migration of the nutrition tube. EN administered by NG or NI tubes seems to provide equal safety,

tolerability and efficacy, even if more results are necessary to validate the routinely use of NG tubes in SAP patients.

COMMENTS

Background

Severe acute pancreatitis (SAP) requires an adequate nutritional support. Enteral nutrition (EN) should be preferred to total parenteral nutrition in patients with SAP, as it is associated with reduced mortality and complications. However, in clinical practice EN is employed far less frequently than it should. The main obstacle to EN diffusion is that it is considered complicated, as to ensure full pancreatic rest, nutrition tubes should be placed in the jejunum, requiring often troublesome procedures. In the past few years, it has been proposed that EN through nasogastric (NG) tubes may be a simple, safe and equally valid alternative to nasojejunal tubes.

Research frontiers

The authors speculated that a pragmatic possibility in real-world clinical practice would be to employ NG feeding whenever tube migration to the jejunum of bedside inserted feeding tubes does not occur spontaneously. They therefore aimed at assessing the rate of spontaneous distal migration of EN tubes in patients with predicted SAP, to identify possible factors associated with it, and to compare the safety and tolerability of EN with an elemental formula in patients who started nutrition with a "proximal", NG or a "distal", naso-intestinal (NI) tube, depending on the success of spontaneous tube migration.

Innovations and breakthroughs

The authors found that spontaneous tube migration to a NI site occurred in 10/25 (40%) prospectively enrolled SAP patients, while in 15 (60%) nutrition was started with a NG tube. Groups were similar for demographics and pancreatitis aetiology but computed tomography (CT) severity index was higher in NG tube patients than in NI (mean 6.2 *vs* 4.7, $P = 0.04$). The CT index seemed a risk factor for failed obtainment of spontaneous distal migration. EN trough NG or NI tube were similar in terms of tolerability, safety, clinical goals, complications and hospital stay.

Applications

This is the first study of its kind observing the outcome of EN in SAP patients in a "real world" clinical setting, with the study protocol driven by the need to have more solid grounds in making clinical decisions about everyday medical care circumstances. Both the proximal and the distal enteral approaches resulted to be feasible, safe and effective in most patients. This issue has a relevant impact on everyday clinical practice as the main limit to EN usage in AP is the technical difficulty in obtaining small bowel access.

Peer review

NG tube insertion, a simpler approach, will probably replace total parenteral nutrition and nasojujunal feeding in the near future. Therefore, despite the small number of patients, this paper is suitable for publication after revision.

REFERENCES

1 **Forsmark CE**, Baillie J. [AGA Institute technical review on acute pancreatitis] *Rev Gastroenterol Mex* 2007; **72**: 257-285
2 **Petrov MS**, Pylypchuk RD, Emelyanov NV. Systematic review: nutritional support in acute pancreatitis. *Aliment Pharmacol Ther* 2008; **28**: 704-712
3 **Al-Omran M**, Albalawi ZH, Tashkandi MF, Al-Ansary LA. Enteral versus parenteral nutrition for acute pancreatitis. *Cochrane Database Syst Rev* 2010; CD002837
4 **Working Party of the British Society of Gastroenterology**; Association of Surgeons of Great Britain and Ireland; Pancreatic Society of Great Britain and Ireland; Association of Upper GI Surgeons of Great Britain and Ireland. UK guidelines for the management of acute pancreatitis. *Gut* 2005; **54** Suppl 3: iii1-iii9
5 **Banks PA**, Freeman ML. Practice guidelines in acute pancreatitis. *Am J Gastroenterol* 2006; **101**: 2379-2400
6 **Pezzilli R**, Uomo G, Zerbi A, Gabbrielli A, Frulloni L, De Rai P, Delle Fave G, Di Carlo V. Diagnosis and treatment of acute pancreatitis: the position statement of the Italian Association for the study of the pancreas. *Dig Liver Dis* 2008; **40**: 803-808
7 **Pezzilli R**, Uomo G, Gabbrielli A, Zerbi A, Frulloni L, De Rai P, Castoldi L, Cavallini G, Di Carlo V. A prospective multicentre survey on the treatment of acute pancreatitis in Italy. *Dig Liver Dis* 2007; **39**: 838-846
8 **Spanier BW**, Mathus-Vliegen EM, Tuynman HA, Van der Hulst RW, Dijkgraaf MG, Bruno MJ. Nutritional management of patients with acute pancreatitis: a Dutch observational multicentre study. *Aliment Pharmacol Ther* 2008; **28**: 1159-1165
9 **O'Keefe SJ**. Physiological response of the human pancreas to enteral and parenteral feeding. *Curr Opin Clin Nutr Metab Care* 2006; **9**: 622-628
10 **Kaushik N**, Pietraszewski M, Holst JJ, O'Keefe SJ. Enteral feeding without pancreatic stimulation. *Pancreas* 2005; **31**: 353-359
11 **DiSario JA**. Endoscopic approaches to enteral nutritional support. *Best Pract Res Clin Gastroenterol* 2006; **20**: 605-630
12 **Karsenti D**, Viguier J, Bourlier P, d'alteroche L, Barbieux JP, Metman EH, Dorval E. Enteral nutrition during acute pancreatitis: feasibility study of a self-propeeling spiral distal end jejunal tube. *Gastroenterol Clin Biol* 2003; **27**: 614-617
13 **Gabriel SA**, Ackermann RJ, Castresana MR. A new technique for placement of nasoenteral feeding tubes using external magnetic guidance. *Crit Care Med* 1997; **25**: 641-645
14 **Heiselman DE**, Hofer T, Vidovich RR. Enteral feeding tube placement success with intravenous metoclopramide administration in ICU patients. *Chest* 1995; **107**: 1686-1688
15 **Eatock FC**, Chong P, Menezes N, Murray L, McKay CJ, Carter CR, Imrie CW. A randomized study of early nasogastric versus nasojejunal feeding in severe acute pancreatitis. *Am J Gastroenterol* 2005; **100**: 432-439
16 **Kumar A**, Singh N, Prakash S, Saraya A, Joshi YK. Early enteral nutrition in severe acute pancreatitis: a prospective randomized controlled trial comparing nasojejunal and nasogastric routes. *J Clin Gastroenterol* 2006; **40**: 431-434
17 **Eckerwall GE**, Axelsson JB, Andersson RG. Early nasogastric feeding in predicted severe acute pancreatitis: A clinical, randomized study. *Ann Surg* 2006; **244**: 959-965; discussion 965-967
18 **Krenitsky J**. Gastric versus jejunal feeding: evidence or emotion? *Pract Gastroenterol* 2006; **30**: 46-65
19 **Berger MM**, Bollmann MD, Revelly JP, Cayeux MC, Pilon N, Bracco D, Chioléro RL. Progression rate of self-propelled feeding tubes in critically ill patients. *Intensive Care Med* 2002; **28**: 1768-1774
20 **Lecleire S**, Antonietti M, Ben-Soussan E, Zenoni M, Savoye G, Goria O, Ducrotté P, Lerebours E. Nasojejunal feeding in patients with severe acute pancreatitis: comparison of endoscopic and self-migration tube placement. *Pancreas* 2007; **35**: 376-378
21 **Joubert C**, Tiengou LE, Hourmand-Ollivier I, Dao MT, Piquet MA. Feasibility of self-propelling nasojejunal feeding tube in patients with acute pancreatitis. *JPEN J Parenter Enteral Nutr* 2008; **32**: 622-624
22 **Jiang K**, Chen XZ, Xia Q, Tang WF, Wang L. Early nasogastric enteral nutrition for severe acute pancreatitis: a systematic review. *World J Gastroenterol* 2007; **13**: 5253-5260
23 **Petrov MS**, Correia MI, Windsor JA. Nasogastric tube feeding in predicted severe acute pancreatitis. A systematic review of the literature to determine safety and tolerance. *JOP* 2008; **9**: 440-448
24 **Vege SS**, Gardner TB, Chari ST, Munukuti P, Pearson RK, Clain JE, Petersen BT, Baron TH, Farnell MB, Sarr MG. Low mortality and high morbidity in severe acute pancreatitis without organ failure: a case for revising the Atlanta classification to include "moderately severe acute pancreatitis". *Am J Gastroenterol* 2009; **104**: 710-715
25 **Greenwood JK**, Lovelace HY, McClave SA. Enteral nutrition in acute pancreatitis: a survey of practices in canadian intensive care units. *Nutr Clin Pract* 2004; **19**: 31-36

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BRIEF ARTICLE

Approach to early-onset colorectal cancer: Clinicopathological, familial, molecular and immunohistochemical characteristics

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Abstract

AIM: To characterize clinicopathological and familial features of early-onset colorectal cancer (CRC) and compare features of tumors with and without microsatellite instability (MSI).

METHODS: Forty-five patients with CRC aged 45 or younger were included in the study. Clinical information, a three-generation family history, and tumor samples were obtained. MSI status was analyzed and mismatch repair genes were examined in the MSI families. Tumors were included in a tissue microarray and an immunohistochemical study was carried out with a panel of selected antibodies.

RESULTS: Early onset CRC is characterized by advanced stage at diagnosis, right colon location, low-grade of differentiation, mucin production, and presence of polyps. Hereditary forms represent at least 21% of cases. Eighty-one percent of patients who died during follow-up showed a lack of expression of cyclin E, which could be a marker of poor prognosis. β-catenin expression was normal in a high percentage of tumors.

CONCLUSION: Early-onset CRC has an important familial component, with a high proportion of tumors showing microsatellite stable. Cyclin E might be a poor prognosis factor.

Key words: Early onset colorectal cancer; Microsatellite instability; Lynch syndrome; Microsatellite stable colorectal cancer

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INTRODUCTION

The prevalence of colorectal cancer (CRC) has been increasing during recent years. In 2004, it had the second highest incidence of all cancers and was the second most common cause of cancer-related death in the European Union[1]. Early onset CRC is infrequent, with an incidence of 2%-8% of all CRCs. In the United States, between 1992 and 2005, the incidence of CRC in young individuals (ages 20-49 years) increased at a rate of 1.5% per year in men and 1.6% per year in women[2].

Early onset of cancer is an indicator that a hereditary component is more likely. The most frequent hereditary form of CRC is Lynch syndrome. It is estimated to represent about 2%-5% of all CRCs, and is characterized by the development of CRC (and other types of cancer) at a mean age of 43 years[3-6]. Its molecular basis is a DNA mismatch repair (MMR) gene defect, which leads to changes in the length of repetitive DNA sequences, known as microsatellite instability (MSI).

The proportion of MSI tumors found in young patients varies between 19.7% and 41%, depending on the age of onset[7-9]. On the other hand, Lynch syndrome is estimated to cause about 1/3 of the CRC cases occurring at a young age[4,10,11]. There are some controversial aspects to the natural history and prognosis of early onset CRC, and some clinical and pathological differences compared to CRC in elderly patients[8]. Early onset CRCs are localized mostly in the right colon, are frequently poorly differentiated, show mucin production, and can develop synchronous and metachronous tumors[12]. These differences are more marked in cases with a family history suggestive of Lynch syndrome, or with molecular characteristics like MSI[8,12-14].

There is little information about microsatellite stable (MSS) forms of CRC in young adults, not only regarding their anatomoclinical features but also regarding their molecular characteristics. For example, there is an increased proportion of MSS tumors in young patients with rectal cancer[15]. Furthermore, several studies show that some alterations in molecular markers typical of MSS early onset CRCs also occur in sporadic cases of CRC, such as modified expression of APC, β-catenin and p53[9].

The aim of our study was to characterize early onset CRC by analyzing its clinical, pathological, familial, molecular, and immunohistochemical (IHC) features. We have determined the proportion of Lynch syndrome in our series, and have compared the characteristics of the MSS and MSI groups.

MATERIALS AND METHODS

Families, samples and data collection

A total of 45 individuals diagnosed with CRC at an age of 45 or younger were collected from two different Spanish institutions (Gregorio Marañón Hospital in Madrid, and Segovia General Hospital). All patients, or a first degree relative in case of death of the index case, provided written consent.

A full three-generation family medical history and colorectal paraffin-embedded tumors were obtained from each proband.

Personal and tumor clinicopathological information was obtained regarding age of onset, gender, location of the CRC (right/left colon or rectum), grade of cell differentiation (low, medium, or high), mucin production, modified Astler-Coller stage, the existence of polyps, and the presence of synchronous or metachronous CRCs. Mean follow-up was 60 mo.

To analyze the familial cancer history of each index case, we divided the neoplasms into two groups: Lynch syndrome-related tumors, and Lynch syndrome-unrelated tumors.

DNA extraction

A tissue specimen was obtained from the index case. Prior to DNA extraction, tumor and normal areas of the paraffin-embedded samples were selected *via* microscopic inspection. The proportion of tumor cells in the material used for DNA extraction exceeded 70% in all cases. DNA was extracted using proteinase K digestion, phenol-chloroform extraction, Phase Lock Gel™ Light (Eppendorf AG, Germany), and EtOH protocol precipitation.

MSI and MMR immunohistochemistry analyses

Microsatellite status was assessed using the BAT-26 mononucleotide marker, based on its high sensitivity[16-18]. However, in order to discard false negative results, all BAT-26 MSS cases fulfilling the Amsterdam I criteria[19], were analyzed using the Bethesda panel. BAT-26 was PCR amplified and fragments were evaluated using an ABI automated sequencer and GeneScan Software. For analysis of the Bethesda panel, we used the HNPCC Microsatellite Instability Test kit (Roche, Mannheim, Germany).

IHC staining for markers of the following processes was performed: MMR; apoptosis; cell adhesion; cell cycle; proliferation, and others. Markers and clones used are shown in Table 1. All samples were fixed onto a tissue microarray, on which the IHC analysis was carried out.

Scoring of tumor staining was done without any knowledge of the patients' family history or results of mutation analyses.

Detection of mutations and large deletions

Cases showing MSI and/or lack of expression of MMR proteins in tumors were screened for germline mutations in the DNA MMR genes *MLH1* and *MSH2*, by denaturing gradient-gel electrophoresis (DGGE) on a DCode System (BioRad), using primers and denaturing conditions previously reported, with slight modifications[20]. Some samples were analyzed by denaturing high-performance liquid chromatography (dHPLC) (http://insertion.stanford.edu/melt.html). When an anomalous band pattern was observed by DGGE or dHPLC, a new PCR product of the fragment was sequenced, using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and analyzed with a Genetic Analyzer ABI Prism 3130 (Applied Biosystems).

Table 1 Antibodies, suppliers and clones used for immunohistochemical analysis

Marker	Supplier	Clone
Mismatch repair system proteins		
Mlh1	BD PharMingen	G168-15
Msh2	Oncogene	FE11
Msh6	BD Transduction Lab.	44
Apoptosis		
Bcl-2	DAKO	124
Cell adhesion		
β-catenin	BD Transduction Lab.	14
E-cadherin	Zymed	4A2C7
Cell cycle markers		
Chk2	Novocastra	DCS 270.1
Cyclin A	Novocastra	6E6
Cyclin D1	NeoMarkers	SP4-rabbit
Cyclin D3	Novocastra	DCS-22
Cyclin E	Novocastra	13A3
p16	Santa Cruz	F-12
p21 (WAF1)	Oncogene	EA10
p27	BD Transduction Lab.	57
RB-P	Santa Cruz	Poli-rabbit
Skp2	Zymed	1G12E9
Cdk2	NeoMarkers	8D4
Proliferation		
p53	Novocastra	DO-7
Ki-67	DAKO	MIB-1
Others		
CK20	DAKO	Ks20.8
RAD50	Abcam	2C6
SMAD4	Santa Cruz	B8

Statistical analysis

Continuous variables were expressed as the median values plus/minus SD, and categorical variables were expressed as number of cases and their percentage. Differences were considered significant when $P < 0.05$. For associations between clinicopathological, familial and molecular features, and MSI status, statistical analyses were performed using Pearson's χ^2 test for parametric variables, and Fisher's exact test for non-parametric variables. When those features were continuous variables, Student's *t*-test was used, as well as for some familial features, to compare the differences between both groups. The SPSS v.11.5 for Windows (SPSS, Inc., Chicago, IL) statistical package was used.

RESULTS

Clinicopathological features

We studied a total of 45 subjects diagnosed with CRC at an age of 45 years old or younger. Clinicopathological characteristics of all cases are shown in Table 2. A high proportion of tumors were located in the right side of the colon (45%). The proportion of poorly differentiated tumors was also notable (16%). The proportion of mucin-producing tumors (mucinous tumors and "signet ring" cell tumors) was 33% of all cases. More than 50% of all tumors were in an advanced stage when diagnosed, with lymph-node involvement and/or distant metastasis. Other remarkable features were incidence of polyps found during follow-up (33%) and three cases (7%) of synchronous and metachronous CRCs. Adenomatous polyps were the most frequently observed type of polyp (13/15).

Median overall survival was 60 mo, while Median Free-Disease Survival was 48 mo. Twenty-three point nine per cent of the patients showed recurrence during follow-up, and overall mortality was 38% (17 patients).

Table 2 Clinical, pathological, and familial characteristics of the global group and the microsatellite stable and microsatellite instability groups *n* (%)

	Global	MSS	MSI	*P* (χ^2)
Patients	45 (100)	29 (69)[1]	13 (31)[1]	
Median age of onset (range, yr)	39 (25-45)	41 (25-45)	37 (32-42)	0.03[2]
Sex				
Male	23 (51)	13 (45)	7 (54)	
Female	22 (49)	16 (55)	6 (46)	NS
Location				
Right colon	20 (45)	11 (38)	9 (69)	
Left colon	15 (33)	11 (38)	2 (16)	
Rectum	10 (22)	7 (24)	2 (15)	NS
Tumor differentiation				
Well	2 (4)	2 (7)	0 (0)	
Moderate	36 (80)	23 (79)	10 (77)	
Poor	7 (16)	4 (14)	3 (23)	NS
Mucin production	15 (33)	5 (17)	5 (39)	NS
"Signet ring" cells	4 (9)	0/29 (0)	4/13 (30)	0.006
Modified Astler Coller stage				
A	3 (7)	2 (7)	0 (0)	
B	17 (38)	12 (41)	5 (39)	
C	15 (33)	7 (24)	6 (46)	
D	10 (22)	8 (28)	2 (15)	NS
Associated polyps during follow-up	15 (33)	10 (35)	4 (31)	NS
Synchronous or metachronous CRCs	3 (7)	1 (3)	1 (8)	NS
Familial history of cancer				
Lynch-related neoplasms in family	18 (40)	5 (17)	11 (85)	< 0.01
Lynch unrelated neoplasms	12 (27)	8 (28)	7 (54)	0.02
Sporadic cases	27 (60)	24 (83)	2 (15)	< 0.001

[1]Proportions were calculated for 42 tumors; [2]Statistical comparison between microsatellite stable (MSS) and microsatellite instability (MSI) groups was performed using Student´s *t* test. CRC: Colorectal cancer; NS: Not significant.

Familial features

Table 2 shows the familial cancer history data. One patient was a case with familial adenomatous polyposis (FAP); 27 (60%) were sporadic cases with Lynch syndrome (without Lynch syndrome-associated neoplasms in their families), while 20% showed familial aggregation, and eight fulfilled Amsterdam II criteria[6]. Apart from CRC, the other most frequent tumors were endometrial adenocarcinoma (six families) and gastric cancer (four families). Twelve families (27%) had other neoplasms not related to Lynch syndrome; the most frequent ones being breast cancer (four cases), larynx cancer (three cases), and leukemia (three cases).

Table 3 Immunohistochemical study of the global group[1] *n* (%)

	Normal expression	Lack of expression
Mismatch repair system proteins		
Mlh1	24 (86)	4 (14)
Msh2	24 (86)	4 (14)
Msh6	23 (88)	3 (12)
Apoptosis		
Bcl-2	7 (25)	21 (75)
Cell adhesion		
β-catenin		
Membrane	24 (86)	4 (14)
Nucleus	10 (36)	18 (64)
E-cadherin	19 (73)	7 (27)
Cell cycle markers		
Chk2	13 (54)	11 (46)
Cyclin A	13 (46)	15 (54)
Cyclin D1	12 (43)	16 (57)
Cyclin D3	12 (43)	16 (57)
Cyclin E	8 (30)	19 (70)
p16	13 (48)	14 (52)
p21	14 (56)	11 (44)
p27	12 (52)	11 (48)
RB-P	13 (48)	14 (52)
Skp2	12 (46)	14 (54)
Cdk2	13 (48)	14 (52)
Proliferation		
p53	15 (54)	13 (46)
Ki-67	17 (63)	6 (37)
Others		
CK20	10 (59)	7 (41)
RAD50	10 (63)	6 (37)
SMAD4	13 (54)	11 (46)

[1]Not all tumors could be studied by immunohistochemical.

Molecular features

Forty-two of the 45 cases were studied for MSI. The three excluded cases were: the FAP case, with an *APC* gene germline mutation (c.916delCT), and two additional cases, due to lack of paraffin embedded tumor tissue. Thirteen tumors (31%) showed MSI and the remaining 29 were therefore defined as MSS.

Blood samples were taken from the MSI index cases for *MLH1* and *MSH2* mutation screening. *MSH6* was not studied because none of the tumors showed lack of Msh6 protein alone in IHC analysis. Eight of the 13 analyzed cases (62%) showed a pathogenic germline mutation in one of the MMR genes: three cases had a mutation in *MLH1*, and five cases had a mutation in *MSH2*. None of the MSS tumors showed lack of expression of MMR proteins in the IHC study.

IHC studies with the remaining antibodies were carried out on 28 cases (Table 3). A remarkable finding was the high proportion of tumors lacking expression of cyclin E, especially in those patients who died during follow-up (9/11). Similarly, all six stage D CRCs included in the IHC study also showed lack of cyclin E expression. The lack of cyclin E expression is either an indicator of poor prognosis or a marker of advanced stage disease. β-catenin and E-cadherin, two proteins in the Wnt pathway, which plays an important role in CRC carcinogenesis, were normal in a high proportion of studied tumors.

Comparison between MSI and MSS tumors

Clinicopathological features: The comparison of the clinicopathological characteristics of the two defined groups (MSS and MSI) is shown in Table 2. Statistically significant differences were observed in the age of onset, which was earlier in the MSI cases, and in the presence of "signet-ring cell" tumors, which were absent in the MSS group. No statistically significant differences were found in the other variables analyzed, probably due to the small sample size. However, it is important to underline that the MSI CRCs were more frequently located proximally (69%), were poorly differentiated with higher mucin production, and were associated with other CRCs. The frequency of polyps during follow-up was the same in MSS and MSI tumors (35% and 31%, respectively). Another remarkable feature was that more than a half of all cases were diagnosed at an advanced stage (with lymph node involvement and/or distant metastasis, stages C or D): 52% for MSS and 61% for MSI.

There might be a trend towards a better prognosis for the MSI group when compared with the MSS group, but without reaching statistical significance: 62 mo *vs* 48 mo for median overall survival, and 62 mo *vs* 29 mo in terms of median disease-free survival. Mortality was higher in the MSS group (41%) than in the MSI group (31%).

Familial features: Table 2 shows familial cancer information and results obtained from the comparison of the MSS and MSI families. MSI cases are more frequently associated to Lynch-related neoplasia. On the other hand, the proportion of sporadic cases is very high in MSS tumors (83%).

Molecular features: The comparison of the IHC study in MSI and MSS tumors is shown in Table 4. None of the MSS tumors demonstrated lack of expression of MMR proteins in the IHC study. There was, as mentioned above, a good correlation between MSI and the IHC study of the MMR proteins.

Normal expression of β-catenin reached the same proportion (86%) in both types of tumors, indicative of the integrity of the Wnt signalling pathway in early age-of-onset CRCs. The high rate of MSI tumors with lack of expression of CK20 and RAD50 was also remarkable.

DISCUSSION

Early onset of CRC in young adults used to be considered to be rare, but many recent reports suggest not only that early onset CRC reaches 8% of all CRCs, but also that it might be increasing[1,2]. Similarly, it is a common belief that early onset CRC is mainly related to hereditary forms, especially to Lynch syndrome. In our study, the presence of a familial background of CRC is confirmed in 38% of the cases, 18% fulfilling Amsterdam criteria type Ⅱ for Lynch syndrome. Hereditary forms of CRC were confirmed in nine patients of the present series. One was a FAP case with an APC germline mutation, and the other eight cases were Lynch syndrome. The rate of MSI in

Table 4 Comparison of the immunohistochemical analyses of microsatellite stable and microsatellite instability groups *n* (%)

	IHC expression				*P*
	MSS		MSI		
	Normal	Lack	Normal	Lack	
MMR system proteins					
Mlh1	20 (100)	0 (0)	4 (50)	4 (50)	0.010
Msh2	21 (100)	0 (0)	3 (43)	4 (57)	0.002
Ms6	21 (100)	0 (0)	2 (40)	3 (60)	0.004
Apoptosis					
Bcl-2	5 (24)	16 (76)	2 (29)	5 (71)	NS
Cell adhesion					
β-catenin					
Membrane	18 (86)	3 (14)	6 (86)	1 (14)	NS
Nucleus	8 (38)	13 (62)	2 (29)	5 (71)	NS
E-cadherin	15 (75)	5 (25)	4 (60)	2 (40)	NS
Cell cycle markers					
Chk2	10 (53)	9 (47)	4 (60)	2 (40)	NS
Cyclin A	8 (38)	13 (62)	5 (71)	2 (29)	NS
Cyclin D1	7 (33)	14 (67)	5 (71)	2 (29)	NS
Cyclin D3	8 (38)	13 (62)	4 (57)	3 (43)	NS
Cyclin E	3 (14)	18 (86)	5 (83)	1 (17)	0.004
p16	9 (43)	12 (57)	4 (67)	2 (33)	NS
p21	10 (53)	9 (47)	4 (67)	2 (33)	NS
p27	8 (44)	10 (56)	4 (80)	1 (20)	NS
RB-P	11 (55)	9 (45)	2 (29)	5 (71)	NS
Skp2	9 (43)	12 (57)	3 (60)	2 (40)	NS
Cdk2	9 (43)	12 (57)	4 (67)	2 (33)	NS
Proliferation					
p53	11 (52)	10 (48)	5 (57)	3 (43)	NS
Ki-67	14 (67)	7 (33)	3 (50)	3 (50)	NS
Others					
CK20	9 (75)	3 (25)	1 (20)	4 (80)	NS
RAD50	9 (75)	3 (25)	1 (25)	3 (75)	NS
SMAD4	11 (55)	9 (45)	2 (50)	2 (50)	NS

IHC: Immunohistochemical; MSS: Microsatellite stable; MSI: Microsatellite instability; MMR: Mismatch repair; NS: Not significant.

our series is similar to that described in previous studies, ranging between 19.7% and 41%[7-9]. Twenty-nine cases showed MSS. This group showed a predominance of sporadic cases. Nevertheless, there were cases that showed a positive family history. Some of them might be associated to Familial CRC type X, namely cases with MSS tumors but fulfilling Amsterdam criteria for Lynch syndrome[21,22]. These findings, however, underline the important, but not unique role, of the known hereditary factors in this age group, prompting further searches for additional causative genes for inherited CRC.

The total sample of early age-of-onset CRCs is characterized by an important proportion of tumors that are localized in the right colon (44%), have a low-grade of differentiation (16%), produce mucin (33%), and have associated polyps (33%). Regarding pathological features, the strong trend towards low-grade differentiation and mucin production of tumors in this age group is described in the literature[7]. Other characteristics, such as synchronous and metachronous CRCs (7%) and the appearance of predominantly adenomatous polyps during the follow-up in a third of the cases, have rarely been studied previously[7,13].

Another finding is the advanced stage of the tumors at diagnosis, with more than half of the cases presenting with lymph node and/or distant metastasis in the pathological exam. This is the consequence of a delay in diagnosis, which is a characteristic of these early age-of-onset CRCs reported repeatedly in the literature[7,8,13].

The results obtained with the cyclin E antibody in the IHC study are quite remarkable. High levels of cyclin E, a G1/S phase transition controller, are found in many different tumors, including CRCs[23-25]. Cyclin E expression has only been evaluated in sporadic forms of CRC, with a variable value as a prognostic factor. Some publications suggest that lack of expression of cyclin E is associated with a faster growth of CRC, but others suggest the opposite[24,26,27]. Seventy per cent of our tumors showed lack of expression of this marker, especially the group of MSS CRCs, in which the proportion reached 86%, while the opposite occurred in the MSI group. The lack of expression of cyclin E might be associated with a poor prognosis, because expression was absent in most patients who died during follow-up (9/11), and all six stage D CRCs included in the IHC study also showed lack of expression of cyclin E. This was observed in both the MSS and the MSI groups, and in the latter group, the only case that showed lack of expression also died during follow-up. Although the sample size is small and the results must therefore be taken with caution, data related to cyclin E as a factor of poor prognosis must be validated in a larger series. In fact, it would be interesting to see if lack of expression of cyclin E corresponds with a subgroup of patients with apparently stable, near-diploid chromosomes and MSS (MACS); CRC in these patients shows an aggressive pattern and the MACS phenotype appears to be overrepresented in early-onset tumors[28,29].

Another important finding of the present study is related to the Wnt pathway. We found a high proportion of normal expression of β-catenin. This protein is an indicator of Wnt pathway dynamics[30], and the expression of β-catenin, mainly in the nucleus, is considered a good marker of the activity of the Wnt pathway[31,32]. The activation of this pathway often occurs in MSS tumors, but also in MSI tumors, independent of the age of onset[33]. Our findings, however, might indicate that the Wnt pathway is not involved in a substantial proportion of our early onset CRCs.

Early onset CRC is a heterogeneous group. To classify different subtypes with common etiology, the use of tools, such as MSI or IHC for MMR proteins, to identify an MMR system deficiency or an intact system (MSS) might be an appropriate and useful approach. All clinicopathological features analyzed in the global series tend to be more marked when the series is divided into MSI and MSS groups. MSI early onset CRCs showed characteristics similar to Lynch syndrome: earlier age of onset, predominant location in the right colon, and a high proportion of poorly differentiated tumors, in accordance with previous reports[8,13]. The same occurs with an elevated proportion of mucin-producing tumors (39%), and "signet-ring" cell tumors. MSS, on the contrary, are tumors that have an

older age of onset, are mainly located in the left colon, and have a low proportion of mucinous or "signet ring" cell tumors.

From the molecular point of view, our data must be confirmed in a larger series to reach more reliable conclusions. Nevertheless, some results should be emphasized. There is a good correlation between the lack of expression of MMR proteins and MSI. This is in agreement with published data, showing a positive prospective value of the IHC of 88%-100%[34,35]. Cyclin E is expressed in most of the MSI tumors, and the opposite occurs in the MSS, as published for sporadic CRC[27]. We found a normal expression of β-catenin in most MSI and MSS tumors. Our results contradict published findings for sporadic CRC, in which a high proportion of MSS tumors show an abnormal expression of β-catenin (90%), which decreases to 65% in MSI tumors[36,37]. There are few published studies focused on early onset CRC but in these the proportion of abnormal β-catenin expression is still significant (77.2% and 42.9% for MSS and MSI, respectively)[8]. Our results, however, should be confirmed by comparing them with control groups of CRCs, to exclude the possibility that technical differences or differences in interpretation of the staining patterns, might explain these contradictory findings.

Early onset CRC has an important proportion of hereditary forms of CRC. The apparent lack of involvement of the Wnt pathway is important, as is the possible value of cyclin E as a poor prognosis factor in early onset CRC. The advanced stage at diagnosis, as well as the still not fully understood group of MSS tumors, should promote a strong effort to diagnose these tumors at an earlier stage, providing a better understanding of MSS early onset CRC.

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COMMENTS

Background

Initially early-onset colorectal cancer (CRC) was thought to be a group mainly composed by hereditary forms of CRC (Lynch syndrome), as early-onset is a characteristic of hereditary forms of cancer, and because of that, most of the publications focused on the hereditary component of this group of CRC.

Research frontiers

There is a larger group of hereditary forms of CRC, compared with that arising in the older population. However, there is an important proportion of tumors that apparently do not show characteristics of the already known hereditary forms of CRC, and which are not well studied.

Innovations and breakthroughs

This is the first time that a complete approach (clinicopathological, familial, molecular, and immunohistochemical) to the early-onset CRC has been performed. The authors have identified certain characteristics that seem to be more frequent in the early-onset CRC. The proportion of hereditary forms, though, represents a relatively small amount of the cases, and some interesting findings are presented that allow prognozing of these patients.

Applications

This is the first step towards a deeper understanding of early-onset CRC, an entity that is increasing in an especially sensitive group of population.

Peer review

The authors present an in depth analysis of young patients (< 45 years) presenting with CRC. Though the sample is small (n = 45) they perform a comparative analysis between those cases that are microsatellite stable and those that show microsatellite instability.

REFERENCES

1 **Boyle P**, Ferlay J. Cancer incidence and mortality in Europe, 2004. *Ann Oncol* 2005; **16**: 481-488
2 **Siegel RL**, Jemal A, Ward EM. Increase in incidence of colorectal cancer among young men and women in the United States. *Cancer Epidemiol Biomarkers Prev* 2009; **18**: 1695-1698
3 **Aaltonen LA**, Salovaara R, Kristo P, Canzian F, Hemminki A, Peltomäki P, Chadwick RB, Kääriäinen H, Eskelinen M, Järvinen H, Mecklin JP, de la Chapelle A. Incidence of hereditary nonpolyposis colorectal cancer and the feasibility of molecular screening for the disease. *N Engl J Med* 1998; **338**: 1481-1487
4 **Samowitz WS**, Curtin K, Lin HH, Robertson MA, Schaffer D, Nichols M, Gruenthal K, Leppert MF, Slattery ML. The colon cancer burden of genetically defined hereditary nonpolyposis colon cancer. *Gastroenterology* 2001; **121**: 830-838
5 **Watson P**, Lynch HT. The tumor spectrum in HNPCC. *Anticancer Res* 1994; **14**: 1635-1639
6 **Vasen HF**, Watson P, Mecklin JP, Lynch HT. New clinical criteria for hereditary nonpolyposis colorectal cancer (HNPCC, Lynch syndrome) proposed by the International Collaborative group on HNPCC. *Gastroenterology* 1999; **116**: 1453-1456
7 **Liang JT**, Huang KC, Cheng AL, Jeng YM, Wu MS, Wang SM. Clinicopathological and molecular biological features of colorectal cancer in patients less than 40 years of age. *Br J Surg* 2003; **90**: 205-214
8 **Losi L**, Di Gregorio C, Pedroni M, Ponti G, Roncucci L, Scarselli A, Genuardi M, Baglioni S, Marino M, Rossi G, Benatti P, Maffei S, Menigatti M, Roncari B, Ponz de Leon M. Molecular genetic alterations and clinical features in early-onset colorectal carcinomas and their role for the recognition of hereditary cancer syndromes. *Am J Gastroenterol* 2005; **100**: 2280-2287
9 **Yiu R**, Qiu H, Lee SH, García-Aguilar J. Mechanisms of microsatellite instability in colorectal cancer patients in different age groups. *Dis Colon Rectum* 2005; **48**: 2061-2069
10 **Aaltonen LA**, Peltomäki P, Mecklin JP, Järvinen H, Jass JR, Green JS, Lynch HT, Watson P, Tallqvist G, Juhola M. Replication errors in benign and malignant tumors from hereditary nonpolyposis colorectal cancer patients. *Cancer Res* 1994; **54**: 1645-1648
11 **Farrington SM**, Lin-Goerke J, Ling J, Wang Y, Burczak JD, Robbins DJ, Dunlop MG. Systematic analysis of hMSH2 and hMLH1 in young colon cancer patients and controls. *Am J Hum* Genet 1998; **63**: 749-759
12 **Chiang JM**, Chen MC, Changchien CR, Chen JS, Tang R, Wang JY, Yeh CY, Fan CW, Tsai WS. Favorable influence of age on tumor characteristics of sporadic colorectal adenocarcinoma: patients 30 years of age or younger may be a distinct patient group. *Dis Colon Rectum* 2003; **46**: 904-910
13 **Gryfe R**, Kim H, Hsieh ET, Aronson MD, Holowaty EJ, Bull SB, Redston M, Gallinger S. Tumor microsatellite instability and clinical outcome in young patients with colorectal cancer. *N Engl J Med* 2000; **342**: 69-77
14 **Chung YF**, Eu KW, Machin D, Ho JM, Nyam DC, Leong AF, Ho YH, Seow-Choen F. Young age is not a poor prognostic marker in colorectal cancer. *Br J Surg* 1998; **85**: 1255-1259
15 **Fernebro E**, Halvarsson B, Baldetorp B, Nilbert M. Predominance of CIN versus MSI in the development of rectal cancer at young age. *BMC Cancer* 2002; **2**: 25
16 **Boland CR**, Thibodeau SN, Hamilton SR, Sidransky D, Eshle-

man JR, Burt RW, Meltzer SJ, Rodriguez-Bigas MA, Fodde R, Ranzani GN, Srivastava S. A National Cancer Institute Workshop on Microsatellite Instability for cancer detection and familial predisposition: development of international criteria for the determination of microsatellite instability in colorectal cancer. *Cancer Res* 1998; **58**: 5248-5257

17 **Hoang JM**, Cottu PH, Thuille B, Salmon RJ, Thomas G, Hamelin R. BAT-26, an indicator of the replication error phenotype in colorectal cancers and cell lines. *Cancer Res* 1997; **57**: 300-303

18 **Loukola A**, Eklin K, Laiho P, Salovaara R, Kristo P, Järvinen H, Mecklin JP, Launonen V, Aaltonen LA. Microsatellite marker analysis in screening for hereditary nonpolyposis colorectal cancer (HNPCC). *Cancer Res* 2001; **61**: 4545-4549

19 **Vasen HF**, Mecklin JP, Khan PM, Lynch HT. The International Collaborative Group on Hereditary Non-Polyposis Colorectal Cancer (ICG-HNPCC). *Dis Colon Rectum* 1991; **34**: 424-425

20 **Wijnen J**, Khan PM, Vasen H, Menko F, van der Klift H, van den Broek M, van Leeuwen-Cornelisse I, Nagengast F, Meijers-Heijboer EJ, Lindhout D, Griffioen G, Cats A, Kleibeuker J, Varesco L, Bertario L, Bisgaard ML, Mohr J, Kolodner R, Fodde R. Majority of hMLH1 mutations responsible for hereditary nonpolyposis colorectal cancer cluster at the exonic region 15-16. *Am J Hum Genet* 1996; **58**: 300-307

21 **Valle L**, Perea J, Carbonell P, Fernandez V, Dotor AM, Benitez J, Urioste M. Clinicopathologic and pedigree differences in amsterdam I-positive hereditary nonpolyposis colorectal cancer families according to tumor microsatellite instability status. *J Clin Oncol* 2007; **25**: 781-786

22 **Boland CR**. Clinical uses of microsatellite instability testing in colorectal cancer: an ongoing challenge. *J Clin Oncol* 2007; **25**: 754-756

23 **Yasui W**, Akama Y, Kuniyasu H, Yokozaki H, Semba S, Shimamoto F, Tahara E. Expression of cyclin E in human gastric adenomas and adenocarcinomas: correlation with proliferative activity and p53 status. *J Exp Ther Oncol* 1996; **1**: 88-94

24 **Yasui W**, Naka K, Suzuki T, Fujimoto J, Hayashi K, Matsutani N, Yokozaki H, Tahara E. Expression of p27Kip1, cyclin E and E2F-1 in primary and metastatic tumors of gastric carcinoma. *Oncol Rep* 1999; **6**: 983-987

25 **Müller-Tidow C**, Metzger R, Kügler K, Diederichs S, Idos G, Thomas M, Dockhorn-Dworniczak B, Schneider PM, Koeffler HP, Berdel WE, Serve H. Cyclin E is the only cyclin-dependent kinase 2-associated cyclin that predicts metastasis and survival in early stage non-small cell lung cancer. *Cancer Res* 2001; **61**: 647-653

26 **Sutter T**, Dansranjavin T, Lubinski J, Debniak T, Giannakudis J, Hoang-Vu C, Dralle H. Overexpression of cyclin E protein is closely related to the mutator phenotype of colorectal carcinoma. *Int J Colorectal Dis* 2002; **17**: 374-380

27 **Bondi J**, Husdal A, Bukholm G, Nesland JM, Bakka A, Bukholm IR. Expression and gene amplification of primary (A, B1, D1, D3, and E) and secondary (C and H) cyclins in colon adenocarcinomas and correlation with patient outcome. *J Clin Pathol* 2005; **58**: 509-514

28 **Chan TL**, Curtis LC, Leung SY, Farrington SM, Ho JW, Chan AS, Lam PW, Tse CW, Dunlop MG, Wyllie AH, Yuen ST. Early-onset colorectal cancer with stable microsatellite DNA and near-diploid chromosomes. *Oncogene* 2001; **20**: 4871-4876

29 **Banerjea A**, Hands RE, Powar MP, Bustin SA, Dorudi S. Microsatellite and chromosomal stable colorectal cancers demonstrate poor immunogenicity and early disease recurrence. *Colorectal Dis* 2009; **11**: 601-608

30 **Morin PJ**, Sparks AB, Korinek V, Barker N, Clevers H, Vogelstein B, Kinzler KW. Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC. *Science* 1997; **275**: 1787-1790

31 **Iwamoto M**, Ahnen DJ, Franklin WA, Maltzman TH. Expression of beta-catenin and full-length APC protein in normal and neoplastic colonic tissues. *Carcinogenesis* 2000; **21**: 1935-1940

32 **Hinoi T**, Tani M, Lucas PC, Caca K, Dunn RL, Macri E, Loda M, Appelman HD, Cho KR, Fearon ER. Loss of CDX2 expression and microsatellite instability are prominent features of large cell minimally differentiated carcinomas of the colon. *Am J Pathol* 2001; **159**: 2239-2248

33 **Shimizu Y**, Ikeda S, Fujimori M, Kodama S, Nakahara M, Okajima M, Asahara T. Frequent alterations in the Wnt signaling pathway in colorectal cancer with microsatellite instability. *Genes Chromosomes Cancer* 2002; **33**: 73-81

34 **Southey MC**, Jenkins MA, Mead L, Whitty J, Trivett M, Tesoriero AA, Smith LD, Jennings K, Grubb G, Royce SG, Walsh MD, Barker MA, Young JP, Jass JR, St John DJ, Macrae FA, Giles GG, Hopper JL. Use of molecular tumor characteristics to prioritize mismatch repair gene testing in early-onset colorectal cancer. *J Clin Oncol* 2005; **23**: 6524-6532

35 **Niessen RC**, Berends MJ, Wu Y, Sijmons RH, Hollema H, Ligtenberg MJ, de Walle HE, de Vries EG, Karrenbeld A, Buys CH, van der Zee AG, Hofstra RM, Kleibeuker JH. Identification of mismatch repair gene mutations in young patients with colorectal cancer and in patients with multiple tumours associated with hereditary non-polyposis colorectal cancer. *Gut* 2006; **55**: 1781-1788

36 **Abdel-Rahman WM**, Ollikainen M, Kariola R, Järvinen HJ, Mecklin JP, Nyström-Lahti M, Knuutila S, Peltomäki P. Comprehensive characterization of HNPCC-related colorectal cancers reveals striking molecular features in families with no germline mismatch repair gene mutations. *Oncogene* 2005; **24**: 1542-1551

37 **Kariola R**, Abdel-Rahman WM, Ollikainen M, Butzow R, Peltomäki P, Nyström M. APC and beta-catenin protein expression patterns in HNPCC-related endometrial and colorectal cancers. *Fam Cancer* 2005; **4**: 187-190

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BRIEF ARTICLE

Is autoimmune hepatitis a frequent finding among HCV patients with intense interface hepatitis?

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Abstract

AIM: To evaluate the overlap of autoimmune hepatitis in hepatitis C virus (HCV)-infected patients with intense interface hepatitis.

METHODS: Among 1759 patients with hepatitis C submitted to liver biopsy, 92 (5.2%) presented intense interface hepatitis. These patients were evaluated regarding the presence of antinuclear antibody (ANA), anti-smooth muscle antibody (SMA) and anti-liver/kidney microsomal antibody (LKM-1), levels of γ-globulin and histological findings related to autoimmune hepatitis (plasma cell infiltrate and presence of rosettes).

RESULTS: Among patients with hepatitis C and intense interface hepatitis there was a low prevalence of autoantibodies (ANA = 12%, SMA = 5%, LKM-1 = 0%) and the median γ-globulin level was within the normal range. Typical histological findings of autoimmune disease were observed in only two cases (2%). After applying the score for diagnosis of autoimmune hepatitis, only one patient was classified with a definitive diagnosis of autoimmune hepatitis. Since overlap with autoimmune hepatitis was not the explanation for the intense necroinflammatory activity in patients with chronic hepatitis C we sought to identify the variables associated with this finding. The presence of intense interface hepatitis was associated with more advanced age, both at the time of infection and at the time of the biopsy, and higher prevalence of blood transfusion and alcohol abuse.

CONCLUSION: Although possible, overlap with autoimmune hepatitis is a very rare association in HCV-infected patients with intense interface hepatitis, an unusual presentation which seems to be related to other host variables.

Key words: Hepatitis C; Liver biopsy; Antinuclear antibody; Autoimmune hepatitis; Interface hepatitis

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INTRODUCTION

Hepatitis C is the main cause of liver-related morbidity and mortality and represents a worldwide public health problem[1]. An estimated 170 million individuals are infected with hepatitis C virus (HCV), corresponding to 3% of the world population[2].

Infection with HCV is characterized by a high chronicity rate (70% to 85%)[3-6], progression to cirrhosis in 20% to 30% of cases[1,6-8] and the development of hepatocarcinoma in 5% of patients[9]. In addition, this infection represents the most common indication for liver transplantation worldwide[10].

Histological analysis of patients chronically infected with HCV usually reveals some degree of fibrosis, generally associated with the presence of mild or moderate necroinflammatory activity[11]. However, a histological pattern demonstrating intense interface hepatitis has been reported[12,13]. In these cases a possible association with autoimmune hepatitis has been suggested, raising doubts regarding the correct diagnosis and the establishment of adequate treatment[14-16]. The objective of the present study was to evaluate the overlap with autoimmune hepatitis in HCV-infected patients with intense interface hepatitis.

MATERIALS AND METHODS

Patients

Patients chronically infected with HCV followed up at the Federal University of Sao Paulo between 1993 and 2006, who were submitted to a liver biopsy, were studied. The inclusion criteria were chronic infection with HCV (characterized by HCV-RNA positivity) and the presence of intense interface hepatitis upon histological analysis. Patients previously treated or who were HBsAg-positive were excluded.

A control group consisting of patients chronically infected with HCV, who presented absent, mild or moderate interface hepatitis, was included in order to evaluate if an eventual association of autoimmune hepatitis with hepatitis C was restricted to patients with intense necroinflammatory activity. In the absence of such association, a comparison with the control group was performed to evaluate other factors possibly related to intense interface hepatitis. This control group was randomly selected from the database of the Hepatitis Outpatient Clinic of the Federal University of Sao Paulo (1:1 ratio). The same exclusion criteria were adopted for the control group. For the comparative analysis, patients with associated diseases [human immunodeficiency virus (HIV), end-stage renal disease and kidney transplant] were excluded from both groups.

The study was approved by the local Ethical Committee.

Epidemiological characteristics

The patients were evaluated regarding gender, age, estimated duration of infection, age at the time of infection, abusive alcohol consumption (men > 40 g/d and women > 20 g/d), the presence of parenteral risk factors (intravenous drug use, hemodialysis or blood transfusion before 1992) and associated diseases (HIV, end-stage renal disease and kidney transplant). This information was recovered from charts where the data were systematically evaluated with a standardized questionnaire. The duration of infection was evaluated in patients with parenteral risk factors and was estimated from the first year of intravenous drug use or hemodialysis or from the year of first transfusion in patients who had received blood transfusions before 1992.

Laboratory tests

The liver enzymes alanine aminotransferase (ALT), aspartate aminotransferase (AST), γ-glutamyltransferase (GGT) and alkaline phosphatase were assayed by an automated kinetic method and were expressed as the following index: value obtained/upper limit of normal. γ-globulins were assayed by electrophoretic fractionation on agarose gel and densitometry. All biochemical tests were performed within a period of 3 mo from the date of the liver biopsy.

Antinuclear antibody (ANA), anti-smooth muscle antibody (SMA), anti-liver/kidney microsomal antibody (anti-LKM) and anti-mitochondrial antibody were determined by indirect immunofluorescence and the titer was considered significant when higher than 1/40.

The patients were tested for the presence of HBsAg and anti-HIV-1/2 using commercial kits (Abbott Laboratories, Chicago, IL, USA). Anti-HCV was determined with a third-generation enzyme immunoassay (Abbott Laboratories, Chicago, IL, USA). Qualitative HCV-RNA was detected by PCR using the Amplicor® Hepatitis C Virus Test, version 2.0 (Roche Molecular Systems, Branchburg, NJ, USA), with a detection limit of 50 IU/mL. HCV genotyping was performed by VERSANT HCV Genotype Assay - LiPA (Innogenetics N.V., Belgium).

Histological analysis

A liver biopsy was indicated in all patients, irrespective of ALT levels. Liver tissue fragments were obtained by percutaneous biopsy with a Tru-cut® needle. The liver biopsy slides were stained with hematoxylin-eosin, Masson's trichrome, Prussian blue (Perls' stain), and silver for reticular fibers (Gomori's stain), and were reviewed by a single pathologist who was unaware of the clinical data. Histological analysis included the determination of the grade of interface hepatitis and of the stage of fibrosis, which were assessed using a semiquantitative scoring system according to Ludwig[17]. Patients were classified as having intense interface hepatitis if they presented a score of periportal activity = 4, in a scale varying from 0 (no inflammation) to 4 (intense necroinflammatory activity).

In order to better characterize the presence of eventual histological components suggestive of autoimmune injury, the presence of plasma cell infiltrate and rosettes was also analyzed.

Scoring system for diagnosis of autoimmune hepatitis

All patients were evaluated regarding the reviewed interna-

Table 1 General characteristics of patients with intense interface hepatitis *n* (%)

	Intense interface hepatitis (*n* = 92)
Male gender	52 (57)
Age (mean ± SD, yr)	49.8 ± 10.5
Age at the time of infection (mean ± SD, yr)	29.5 ± 9.8
Parenteral risk factor	59 (64)
Duration of infection (mean ± SD, yr)	20.1 ± 8.7
History of blood transfusion	44 (48)
Intravenous drug use	13 (14)
Hemodialysis	2 (2)
Alcoholism	25 (27)
Human immunodeficiency virus-positive	5 (5)
Renal transplant	6 (7)
End-stage renal disease	2 (2)
Alanine aminotransferase (xULN)	4.1 (0.3-18.2)
Aspartate aminotransferase (xULN)	3.0 (0.9-10.4)
γ-glutamyltransferase (xULN)	4.1 (0.1-16.4)
Alkaline phosphatase (xULN)	0.9 (0.3-3.8)
γ-globulin (g/dL)	1.9 (0.73-5.74)
Antinuclear antibody	11 (12)[1]
Anti-smooth muscle antibody	5 (5)
Anti-liver/kidney microsomal antibody	0 (0)
Antimitochondrial antibody	1 (1)
Hepatitis C virus genotype	
Genotype 1	53/77 (69)
Genotype non-1	24/77 (31)
Cirrhosis	53 (58)
Parenchymatous activity ⩾ 3	50 (54)
Intense plasma cell infiltrate	2 (2)
Rosettes	26 (28)

[1]All antinuclear antibody positive patients presented the speckled pattern; titers varied from 1/80 to 1/640. xULN: Times the upper limit of normal.

tional diagnostic criteria for autoimmune hepatitis according to the International Autoimmune Hepatitis Group[18].

Statistical analysis

The χ^2 test and Fisher's exact test were used for statistical analysis of categorical variables. Numerical variables were compared between the two groups using the Student *t*-test and Mann-Whitney test. A level of significance of 0.05 (α = 5%) was adopted.

RESULTS

Among the 1759 patients chronically infected with HCV submitted to a liver biopsy during the study period, 92 presented intense interface hepatitis, corresponding to 5.2% of the initial sample. The characteristics of these patients are shown in Table 1.

Among patients presenting intense interface hepatitis, there was a low prevalence of autoantibodies and the median γ-globulin level was within the normal range. Typical histological findings of autoimmune disease were observed in only two cases (2%). After applying the scoring system for diagnosis of autoimmune hepatitis only one patient was classified as having a definitive diagnosis.

Since overlap with autoimmune hepatitis was not the explanation for the intense necroinflammatory activity in patients with chronic hepatitis C, we sought to identify the variables associated with this finding. Therefore, we compared epidemiological, laboratory and histological characteristics between patients with intense interface hepatitis and a randomly selected control group consisting of chronic HCV-infected patients with absent, mild or moderate interface hepatitis. For comparison between groups, 13 patients with associated disease were excluded from the group with intense interface hepatitis: 6 patients with kidney transplant, 5 with HIV co-infection and 2 with end-stage renal disease.

Table 2 Comparative analysis of general characteristics between groups *n* (%)

	Group 1 (*n* = 79)	Group 2 (*n* = 79)	*P*
Male gender	44 (56)	49 (62)	0.42
Mean age (mean ± SD, yr)	50.8 ± 10.6	43.9 ± 11.5	< 0.001
Alcoholism	22 (28)	10 (13)	0.02
Blood transfusion	37 (47)	23 (29)	0.02
Intravenous drug use	10 (13)	9 (11)	0.81
Duration of infection (mean ± SD, yr)	22.2 ± 7.9	20.9 ± 7.5	0.49
Age at the time of infection (mean ± SD, yr)	28.6 ± 9.6	23.0 ± 11.2	0.02
ALT (xULN)	4.2 (0.3-18.2)	1.8 (0.9-11.3)	< 0.001
AST (xULN)	3.1 (0.9-10.4)	1.4 (0.8-5.8)	< 0.001
GGT (xULN)	3.8 (0.1-16.4)	1.1 (0.1-10.6)	< 0.001
Alkaline phosphatase (xULN)	0.8 (0.3-2.1)	1.0 (0.1-3.0)	0.32
γ-globulin (g/dL)	1.9 (0.7-4.0)	1.7 (0.9-3.7)	0.19
ANA positive	9 (11)	7 (9)	0.59
Genotype 1	43/65 (66)	54/79 (68)	0.78
Cirrhosis	50 (63)	20 (25)	< 0.001
Parenchymatous activity ⩾ 3	43 (55)	3 (4)	< 0.001

Group 1: Patients with intense interface hepatitis; Group 2: Patients with absent, mild or moderate interface hepatitis. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GGT: γ-glutamyltransferase; xULN: Times the upper limit of normal; ANA: Antinuclear antibody.

In the group of patients with intense interface hepatitis, the subjects were older and the proportions of blood transfusion and abusive alcohol consumption were higher. In addition, these patients presented higher levels of ALT (4.2 *vs* 1.8, $P < 0.001$), AST (3.1 *vs* 1.4, $P < 0.001$) and GGT (3.8 *vs* 1.1, $P < 0.001$). No difference in the proportion of patients with reactive ANA or serum γ-globulin levels was observed between groups (Table 2).

Regarding liver biopsy, the mean number of portal tracts observed was 11. Histological aspects are presented in Table 2. The proportion of patients with moderate to intense lobular necroinflammatory activity and cirrhosis was higher in the group with intense interface hepatitis ($P < 0.001$).

DISCUSSION

Previous studies have demonstrated that the presence of intense interface hepatitis in patients chronically infected with HCV is rare[19,20]. When this finding is present, other liver diseases, especially autoimmune hepatitis, should be carefully ruled out. In the present study, 1759 patients

chronically infected with HCV were initially evaluated and in 92 of them (5.2%) a liver biopsy revealed intense interface hepatitis, indicating that, although uncommon, this finding might be a histological pattern of hepatitis C.

The main objective of the present study was to evaluate the overlap with autoimmune hepatitis in HCV-infected patients with intense interface hepatitis. In this sample only two patients (2%) had serological and histological evidence of autoimmunity in the group with intense interface hepatitis and only one patient had a definitive diagnosis of autoimmune hepatitis based on the International Autoimmune Hepatitis Group scoring system[18]. Although a 12% prevalence of ANA was found among the intense interface hepatitis patients, there was no difference in the proportion of patients with positive ANA when they were compared to patients with less intense necroinflammatory activity. In addition, the prevalence of SMA and anti-LKM was very low in the group with intense interface hepatitis.

No histological lesions typical of autoimmune hepatitis were identified in all except two patients and the proportion of cases presenting a significant plasma cell infiltrate was very low in patients with intense interface hepatitis. The high proportion of patients with rosettes observed in the group with intense interface hepatitis was not considered as suggestive of autoimmune injury, since it reflects hepatic regeneration activity as a consequence of greater necroinflammatory activity and can be observed in other etiologies of liver disease[21,22]. These findings support the suggestion that overlap with autoimmune hepatitis is a very rare association in HCV-infected patients with intense interface hepatitis and raises the possibility that some mechanism related to the host-virus interaction might be responsible for the intense interface hepatitis observed.

Since overlap with autoimmune hepatitis was not found in association with intense necroinflammatory activity in patients with chronic hepatitis C we sought to identify other variables associated with this finding.

In comparison to the control group, the presence of intense interface hepatitis was associated with the following epidemiological characteristics: more advanced age both at the time of infection and at the time of the biopsy, and a higher prevalence of blood transfusion and alcohol abuse. With respect to age at the time of infection, a higher necroinflammatory hepatic activity was observed in patients with more advanced age at HCV infection[19,23]. However, the mechanisms related to this phenomenon are still unknown. One hypothesis is that the ability of the immune system to contain the pathological process triggered by the HCV infection declines with age. It is possible that the higher proportion of patients with a history of blood transfusion in the group with intense interface hepatitis, another association observed in this study, also reflects the association between more advanced age and intense interface hepatitis, since in this sample patients with a history of transfusion were older ($P = 0.025$).

Excessive alcohol consumption was another variable associated with intense interface hepatitis, suggesting that alcohol may modify the histological injury induced by HCV[23,24], rendering the disease more aggressive even in the absence of lesions characteristic of direct alcoholic hepatic disease. The mechanism whereby alcohol may aggravate the HCV-induced inflammatory process remains obscure.

Analysis of biochemical and histological characteristics demonstrated that patients with intense interface hepatitis present with more severe liver disease, including a high proportion of cirrhosis (63%). With respect to liver enzymes, significantly higher ALT, AST and GGT levels were observed, an expected finding since elevated aminotransferases[25] and GGT[26] levels have been shown to be associated with greater hepatic inflammatory activity.

Although an association between genotype 1 and more intense necroinflammatory activity has been demonstrated[27], no such association between HCV genotype and severity of liver disease was observed in the present study and in most of the studies reported in the literature[28-32].

Regarding histological findings, the histological variables associated with intense interface hepatitis were advanced fibrosis and more intense parenchymatous activity. Although the association between necroinflammatory activity and fibrosis is controversial, this finding supports the hypothesis that necroinflammatory activity influences the progression of hepatic fibrosis as demonstrated in other studies[33-36]. The parenchymatous activity was another variable independently associated with intense interface hepatitis. Although the interface hepatitis is the main histological lesion observed in chronic hepatitis C, whenever the necroinflammatory activity is intense, this process tends to involve all the compartments, and is not restricted to the portal tract.

In conclusion, the absence of elevated γ-globulin levels, the low prevalence of autoantibodies, the occurrence of typical histological findings of autoimmune disease in only two cases (2%), and a definitive diagnosis according to the autoimmune hepatitis score in only one case, suggest that overlap with autoimmune hepatitis is a very rare association in HCV-infected patients with intense interface hepatitis. The uncommon histological presentation of hepatitis C with intense interface hepatitis seems to be related mainly to other host variables.

COMMENTS

Background

Previous studies have demonstrated that intense interface hepatitis is an uncommon finding in chronic hepatitis C. When this finding is present, it raises doubt regarding a possible association with autoimmune hepatitis.

Research frontiers

The main objective of the present study was to evaluate the overlap with autoimmune hepatitis in hepatitis C virus (HCV)-infected patients with intense interface hepatitis.

Innovations and breakthroughs

This study demonstrated that overlap with autoimmune hepatitis is a very rare association in HCV-infected patients with intense interface hepatitis. This finding raises the possibility that some mechanism related to the host-virus interaction might be responsible for this histological pattern.

Applications

Considering that overlap with autoimmune hepatitis in HCV-infected patients with intense interface hepatitis is very uncommon, the best clinical approach for these patients should be antiviral therapy. These results reduce the dilemma of

whether immunosuppressive therapy is indicated for patients presenting with this histological finding.

Terminology

Interface hepatitis is a histological finding in liver biopsies observed in chronic hepatitis. It is also termed necroinflammatory periportal activity and was formerly known as piecemeal necrosis. Interface hepatitis is graded as mild, moderate or intense. In this study the authors aimed to evaluate HCV-infected patients with intense interface hepatitis.

Peer review

The paper is well written and represents timely research aimed at identifying a link between hepatitis C and autoimmune hepatitis.

REFERENCES

1 **Alberti A**, Benvegnù L. Management of hepatitis C. *J Hepatol* 2003; **38** Suppl 1: S104-S118
2 **National Institutes of Health**. National Institutes of Health Consensus Development Conference Statement: Management of hepatitis C: 2002--June 10-12, 2002. *Hepatology* 2002; **36**: S3-S20
3 **Hoofnagle JH**. Hepatitis C: the clinical spectrum of disease. *Hepatology* 1997; **26**: 15S-20S
4 **Alter MJ**, Kruszon-Moran D, Nainan OV, McQuillan GM, Gao F, Moyer LA, Kaslow RA, Margolis HS. The prevalence of hepatitis C virus infection in the United States, 1988 through 1994. *N Engl J Med* 1999; **341**: 556-562
5 **Villano SA**, Vlahov D, Nelson KE, Cohn S, Thomas DL. Persistence of viremia and the importance of long-term follow-up after acute hepatitis C infection. *Hepatology* 1999; **29**: 908-914
6 **Alter HJ**, Seeff LB. Recovery, persistence, and sequelae in hepatitis C virus infection: a perspective on long-term outcome. *Semin Liver Dis* 2000; **20**: 17-35
7 **Lauer GM**, Walker BD. Hepatitis C virus infection. *N Engl J Med* 2001; **345**: 41-52
8 **Seeff LB**. Natural history of chronic hepatitis C. *Hepatology* 2002; **36**: S35-S46
9 **Tong MJ**, el-Farra NS, Reikes AR, Co RL. Clinical outcomes after transfusion-associated hepatitis C. *N Engl J Med* 1995; **332**: 1463-1466
10 **Seaberg EC**, Belle SH, Beringer KC, Schivins JL, Detre KM. Liver transplantation in the United States from 1987-1998: updated results from the Pitt-UNOS Liver Transplant Registry. *Clin Transpl* 1998; 17-37
11 **Járnay K**, Karácsony G, Ozsvár Z, Nagy I, Lonovics J, Schaff Z. Assessment of histological features in chronic hepatitis C. *Hepatogastroenterology* 2002; **49**: 239-243
12 **Roudot-Thoraval F**, Bastie A, Pawlotsky JM, Dhumeaux D. Epidemiological factors affecting the severity of hepatitis C virus-related liver disease: a French survey of 6,664 patients. The Study Group for the Prevalence and the Epidemiology of Hepatitis C Virus. *Hepatology* 1997; **26**: 485-490
13 **Pasquale G**, Sagnelli E, Coppola N, Onofrio M, Scarano F, Scolastico C, Bellomo PF, Lettieri A, Mogavero AR, Caprio N, Sagnelli C, Piccinino F. [An attempt to improve classification of HCV-correlated chronic hepatitis] *Infez Med* 2005; **13**: 16-22
14 **Cassani F**, Cataleta M, Valentini P, Muratori P, Giostra F, Francesconi R, Muratori L, Lenzi M, Bianchi G, Zauli D, Bianchi FB. Serum autoantibodies in chronic hepatitis C: comparison with autoimmune hepatitis and impact on the disease profile. *Hepatology* 1997; **26**: 561-566
15 **Czaja AJ**, Carpenter HA. Histological findings in chronic hepatitis C with autoimmune features. *Hepatology* 1997; **26**: 459-466
16 **Antonaci S**, Giannelli G, Simone B, Vella FS. [Syndrome of overlap: Chronic hepatitis C/autoimmune hepatitis: fact or fancy?] *Recenti Prog Med* 2005; **96**: 27-31
17 **Ludwig J**. The nomenclature of chronic active hepatitis: an obituary. *Gastroenterology* 1993; **105**: 274-278
18 **Alvarez F**, Berg PA, Bianchi FB, Bianchi L, Burroughs AK, Cancado EL, Chapman RW, Cooksley WG, Czaja AJ, Desmet VJ, Donaldson PT, Eddleston AL, Fainboim L, Heathcote J, Homberg JC, Hoofnagle JH, Kakumu S, Krawitt EL, Mackay IR, MacSween RN, Maddrey WC, Manns MP, McFarlane IG, Meyer zum Büschenfelde KH, Zeniya M. International Autoimmune Hepatitis Group Report: review of criteria for diagnosis of autoimmune hepatitis. *J Hepatol* 1999; **31**: 929-938
19 **Delladetsima JK**, Rassidakis G, Tassopoulos NC, Papatheodoridis GV, Smyrnoff T, Vafiadis I. Histopathology of chronic hepatitis C in relation to epidemiological factors. *J Hepatol* 1996; **24**: 27-32
20 **Sood A**, Midha V, Sood N, Kaur H, Malhotra V, Awasthi G. Chronic hepatitis C in northern India--the pathological and clinical spectrum. *J Assoc Physicians India* 2004; **52**: 380-384
21 **Bach N**, Thung SN, Schaffner F. The histological features of chronic hepatitis C and autoimmune chronic hepatitis: a comparative analysis. *Hepatology* 1992; **15**: 572-577
22 **Czaja AJ**, Carpenter HA. Sensitivity, specificity, and predictability of biopsy interpretations in chronic hepatitis. *Gastroenterology* 1993; **105**: 1824-1832
23 **Gomes de Sá Ribeiro Mde F**, da Costa Gayotto LC, de Alencar Fischer Chamone D, Strauss E. Alcoholic intake predisposes to more interface hepatitis in chronic hepatitis C. *Ann Hepatol* 2005; **4**: 176-183
24 **Hézode C**, Lonjon I, Roudot-Thoraval F, Pawlotsky JM, Zafrani ES, Dhumeaux D. Impact of moderate alcohol consumption on histological activity and fibrosis in patients with chronic hepatitis C, and specific influence of steatosis: a prospective study. *Aliment Pharmacol Ther* 2003; **17**: 1031-1037
25 **Pradat P**, Alberti A, Poynard T, Esteban JI, Weiland O, Marcellin P, Badalamenti S, Trépo C. Predictive value of ALT levels for histologic findings in chronic hepatitis C: a European collaborative study. *Hepatology* 2002; **36**: 973-977
26 **Silva IS**, Ferraz ML, Perez RM, Lanzoni VP, Figueiredo VM, Silva AE. Role of gamma-glutamyl transferase activity in patients with chronic hepatitis C virus infection. *J Gastroenterol Hepatol* 2004; **19**: 314-318
27 **Svirtlih N**, Delic D, Simonovic J, Jevtovic D, Dokic L, Gvozdenovic E, Boricic I, Terzic D, Pavic S, Neskovic G, Zerjav S, Urban V. Hepatitis C virus genotypes in Serbia and Montenegro: the prevalence and clinical significance. *World J Gastroenterol* 2007; **13**: 355-360
28 **Booth JC**, Foster GR, Levine T, Thomas HC, Goldin RD. The relationship of histology to genotype in chronic HCV infection. *Liver* 1997; **17**: 144-151
29 **Adinolfi LE**, Utili R, Andreana A, Tripodi MF, Rosario P, Mormone G, Ragone E, Pasquale G, Ruggiero G. Relationship between genotypes of hepatitis C virus and histopathological manifestations in chronic hepatitis C patients. *Eur J Gastroenterol Hepatol* 2000; **12**: 299-304
30 **Mohsen AH**. The epidemiology of hepatitis C in a UK health regional population of 5.12 million. *Gut* 2001; **48**: 707-713
31 **Poynard T**, Ratziu V, Charlotte F, Goodman Z, McHutchison J, Albrecht J. Rates and risk factors of liver fibrosis progression in patients with chronic hepatitis c. *J Hepatol* 2001; **34**: 730-739
32 **Silva GF**, Nishimura NF, Coelho KI, Soares EC. Grading and staging chronic hepatitis C and its relation to genotypes and epidemiological factors in Brazilian blood donors. *Braz J Infect Dis* 2005; **9**: 142-149
33 **Yano M**, Kumada H, Kage M, Ikeda K, Shimamatsu K, Inoue O, Hashimoto E, Lefkowitch JH, Ludwig J, Okuda K. The long-term pathological evolution of chronic hepatitis C. *Hepatology* 1996; **23**: 1334-1340
34 **Fontaine H**, Nalpas B, Poulet B, Carnot F, Zylberberg H, Brechot C, Pol S. Hepatitis activity index is a key factor in determining the natural history of chronic hepatitis C. *Hum Pathol* 2001; **32**: 904-909
35 **Ghany MG**, Kleiner DE, Alter H, Doo E, Khokar F, Promrat K, Herion D, Park Y, Liang TJ, Hoofnagle JH. Progression of fibrosis in chronic hepatitis C. *Gastroenterology* 2003; **124**: 97-104
36 **Lagging LM**, Westin J, Svensson E, Aires N, Dhillon AP, Lindh M, Wejstål R, Norkrans G. Progression of fibrosis in untreated patients with hepatitis C virus infection. *Liver* 2002; **22**: 136-144

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BRIEF ARTICLE

Capecitabine with radiation is an effective adjuvant therapy in gastric cancers

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Abstract

AIM: To analyze the outcome of patients who received concurrent capecitabine (Xeloda) and radiation (XRT) compared to the established concurrent 5-fluorouracil (5-FU) with radiation (5FU-RT) and fluoropyrimidine-based chemotherapy alone as adjuvant treatment in gastric cancers.

METHODS: All patients with gastric cancers who received adjuvant treatment at the National Cancer Centre Singapore between 1996 and 2006 were reviewed. Treatment outcomes of patients who received XRT were compared with those who had 5FU-RT or chemotherapy alone as adjuvant therapy for gastric cancers.

RESULTS: A total of 108 patients were reviewed. Median age at diagnosis was 60. The majority of the patients (64.8%) had advanced stage Ⅲ and Ⅳ disease (with no distant metastasis). All except 4 patients had D2 gastrectomy. Twenty one patients (19.4%) had positive surgical resection margins. Thirty three patients received XRT compared with 52 who had 5FU-RT and 23 who received chemotherapy alone. For the patients in the chemotherapy-only group, all had fluoropyrimidine-based therapy, with added cisplatin in 7 patients and epirubicin in 2 patients. Median recurrence-free survival was longer for the XRT group (52 mo) compared to the 5FU-RT (35 mo) and chemotherapy-only groups (25 mo) (P = 0.48). The patients in the XRT group achieved similar median overall survival (53 mo) as the 5FU-RT (54 mo) and the chemotherapy-only groups (44 mo) (P = 0.5).

CONCLUSION: Capecitabine with concurrent radiation was as effective as concurrent 5FU with radiation or fluoropyrimidine-based chemotherapy alone when used as adjuvant treatment in patients with gastric cancers.

Key words: Capecitabine; Radiation; Gastric cancer; Adjuvant chemotherapy

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INTRODUCTION

Surgery is the curative treatment for gastric cancer. However, the outcome of large T3-T4 tumors and those with lymph node involvement remains poor after surgical resection alone with high risk of local and distant recurrence[1]. Many attempts had been made to improve the prognosis of resected gastric cancers. These include postoperative adjuvant chemotherapy with or without radiation and perioperative chemotherapy.

Adjuvant therapy in gastric cancer is a field of ongoing active research. The different modalities in the adjuvant setting include chemoradiation and chemotherapy alone. There is currently no randomized phase Ⅲ trial that directly compared these various modalities. Hence, the optimal adjuvant therapy in gastric cancer remains to be determined.

Capecitabine (Xeloda), an oral fluoropyrimidine, was shown to have equivalent efficacy to continuous-infusional 5-fluorouracil (5-FU) in several phase Ⅲ trials in metastatic gastric cancers. The REAL-2 study compared capecitabine or 5-FU in combination with cisplatin and oxaliplatin and showed no differences in response rates or survival between all the 4 combination regimens[2]. The ML 17032 trial comparing the combination of capecitabine with cisplatin to 5-FU with cisplatin showed that capecitabine and cisplatin had a better response rate (41% *vs* 29%) and overall survival (10.5 mo *vs* 9.3 mo)[3]. Because of the convenient oral administration of capecitabine, its use in the adjuvant setting is an attractive option to explore. In fact, capecitabine with concurrent radiation as an adjuvant or neoadjuvant treatment in resected gastric cancer and other gastrointestinal malignancies, particularly rectal cancer, has been explored in several studies[4-6]. These studies demonstrated that capecitabine with concurrent radiation was feasible with manageable toxicities. However, the regimen has never been compared with the more established concurrent 5-FU and radiation (5FU-RT) or adjuvant chemotherapy alone.

Hence, in this study, we aimed to analyze the outcome of patients who received adjuvant concurrent capecitabine and radiation (XRT) compared to those who received concurrent FU-RT or fluoropyrimidine-based chemotherapy alone in the adjuvant setting in gastric cancers.

MATERIALS AND METHODS

All patients diagnosed with gastric cancer who received adjuvant treatment at National Cancer Center Singapore from 1996 to 2007 were reviewed. Clinical information was collected retrospectively and included age, gender, performance status, *Helicobacter pylori* status, surgical outcome, tumor histology, carcinoembryonic antigen, baseline hematologic and biochemical parameters, recurrence and survival data.

The patients were divided into 3 groups based on the adjuvant treatment received. These included 5FU-RT, XRT and chemotherapy alone for analysis. The patients in the XRT group received 825 mg/m^2 capecitabine daily with concurrent radiation followed by capecitabine 2000 mg/m^2 every 3 wk for 6 cycles. Patients in the 5FU-RT group received chemoradiation which consisted of 5 cycles of 5-FU 400 mg/m^2 plus leucovorin (20 mg/m^2) given every 28 d with 2 cycles given concurrently with radiation. The total radiation dose in both the XRT and 5FU-RT groups was 45 Gy delivered over a period of 5 wk. The patients in the chemotherapy-only group received fluoropyrimidine-based chemotherapy.

The patients receiving XRT were compared with those who had 5FU-RT and fluoropyrimidine-based chemotherapy in terms of recurrence-free survival and overall survival. We also compared the patients who received radiation as part of their adjuvant therapy to those who received chemotherapy alone.

Comparison of the median age at baseline between treatment groups was done using the Mann-Whitney *U* test. Other baseline characteristics, such as gender, performance status, stage and grade, as shown in Table 1, were performed using Fisher's exact test.

Recurrence-free survival duration was calculated from the date of diagnosis to the date of recurrence or death (whichever occurred first) or last follow-up. Overall survival duration was calculated from the date of diagnosis to the date of death or last follow-up. The Kaplan-Meier method was used to estimate and plot the recurrence-free survival and overall survival curves for the 3 treatment groups. The log-rank test was used to test if the survival function for the treatment groups were statistically different at the 5% significance level.

For recurrence-free survival and overall survival, the Cox proportional hazards model was used to estimate the crude and age-adjusted hazard ratios between treatment groups using the XRT arm as the reference treatment. Age at diagnosis was included in multivariate Cox regression analyses to estimate the adjusted hazard ratios. Subgroup analysis of the recurrence-free survival and overall survival hazard ratios between the chemotherapy-only group and chemoradiotherapy (XRT or 5FU-RT) were performed using the Cox proportional hazard model for patients with a positive surgical margin, Stage 3 or 4, and positive nodes, respectively.

Statistical analysis

The Cox proportional hazards model and log-rank test were also used to estimate the hazard ratios to assess if any of the baseline characteristics were associated with recurrence-free survival and overall survival in each of the 3 treatment groups. Normality of the variables in the Cox model was assessed using a Q-Q plot and proportionality assumption of the Cox model was assessed using the Schoenfeld test.

Two-sided *P*-values of less than 5% were considered as statistically significant. The software used for the analyses was STATA version 9.1.

RESULTS

A total of 108 patients had received adjuvant therapy for gastric cancer at our institution in the specified period. Median follow-up duration was 23 mo.

Table 1 Patient characteristics *n* (%)

	All patients (*n* = 108)	XRT (*n* = 33)	5FU-RT (*n* = 52)	Chemo alone (*n* = 23)	*P*-value[1] for XRT *vs*	
					5FU-RT	Chemo alone
Age (yr)						
Median	60	64	57	56	0.003[2]	0.03[2]
Inter-quartile range	49.5-66.0	57.7-68.8	48.0-63.7	49.3-65.7		
Gender						
Male	65 (60.2)	22 (66.7)	30 (57.7)	13 (56.5)	0.5	0.6
Female	43 (39.8)	11 (33.3)	22 (42.3)	10 (43.5)		
ECOG						
0	6 (5.6)	1 (3.0)	3 (5.8)	2 (8.7)	1.0	0.6
1	102 (94.4)	32 (97.0)	49 (94.2)	21 (91.3)		
Helicobacter pylori status						
Yes	36 (53.7)	16 (66.7)	15 (50.0)	5 (38.5)	0.3	0.2
No	31 (46.3)	8 (33.3)	15 (50.0)	8 (61.5)		
Surgical margins						
Positive	21 (19.4)	4 (12.1)	14 (26.9)	3 (13.0)	0.2	1.0
Negative	87 (80.6)	29 (87.9)	38 (73.1)	20 (87.0)		
Stage						
1 or 2	38 (35.2)	12 (36.4)	14 (26.9)	12 (52.2)	0.5	0.3
3 or 4	70 (64.8)	21 (63.6)	38 (73.1)	11 (47.8)		
Grade						
1 or 2	31 (28.7)	12 (36.4)	12 (23.1)	7 (30.4)	0.2	0.8
3	77 (71.3)	21 (63.6)	40 (76.9)	16 (69.6)		
Albumin						
< 30	12 (13.3)	4 (12.9)	4 (10.0)	4 (21.1)	0.7	0.5
⩾ 30	78 (86.7)	27 (87.1)	36 (90.0)	15 (78.9)		
CEA						
Normal	50 (90.9)	17 (89.5)	24 (92.3)	9 (90.0)	1.0	1.0
High	5 (9.1)	2 (10.5)	2 (7.7)	1 (10.0)		
Hemoglobin						
< 10	26 (25.7)	8 (24.2)	14 (29.2)	4 (20.0)	0.8	1.0
⩾ 10	75 (74.3)	25 (75.8)	34 (70.8)	16 (80.0)		

[1]All *P*-values calculated using Fisher's exact test unless otherwise stated; [2]*P*-value calculated using the Mann-Whitney *U* test. XRT: Capecitabine + radiation; 5FU-RT: 5-fluorouracil + radiation; ECOG: Eastern Cooperative Oncology Group status; CEA: Carcinoembryonic antigen.

Thirty-three of these patients received XRT, 52 received 5FU-RT and 23 received fluoropyrimidine-based chemotherapy. Of the patients who received chemotherapy alone, 11 had capecitabine alone, 6 had ECF (epirubicin, cisplatin and 5-FU), 4 had XELOX (capecitabine and oxaliplatin) and 2 had 5-FU alone. Ninety-one percent of the patients in the XRT group, 89% in the 5FU-RT and 91% in the chemotherapy-only group completed the full course of adjuvant treatment.

The characteristics of these patients are shown in Table 1. Median age at diagnosis was 60 years. Sixty-five percent of the patients had advanced stage Ⅲ and Ⅳ (with no distant metastasis) disease. All except 4 patients had D2 gastrectomy (2 in the XRT group, 2 in the chemotherapy-only group). Twenty-one of these patients (19.4%) had positive surgical resection margins (14 in the 5FU-RT group, 4 in the XRT group and 3 in the chemotherapy-only group). The only significant difference in characteristics among the 3 groups of patients was median age, with the median age of the patients in the XRT group being greater than that of the other 2 groups.

Median recurrence-free survival was longer for the XRT group (52 mo) compared with the 5FU-RT (35 mo) and chemotherapy-only groups (25 mo). However, the recurrence-free survival curves between the 3 groups were not statistically significant ($P = 0.5$, Figure 1A, Table 2). Patients with positive surgical margins were found to have a significantly poorer recurrence-free survival in both the XRT and 5FU-RT groups but not in the chemotherapy-only group (Table 3).

The overall survival times of the patients in the 3 treatment groups were not statistically different ($P = 0.5$), with the median overall survival of the patients in the XRT group at 53 mo, the 5FU-RT group at 54 mo and the chemotherapy-only group at 44 mo (Figure 1B). Patients with positive surgical margins were found to have poorer survival across all the treatment groups (Table 3).

When comparing patients who received radiation as part of their adjuvant therapy to those who received chemotherapy alone, there was no statistical difference in recurrence-free survival and overall survival between these 2 groups of patients (Figure 1C and D). Subgroup analyses of patients with positive surgical margins, lymph node positive and T3 or T4 disease did not show a statistically difference in survival between patients who received radiation as part of their adjuvant therapy and those who did not (Table 4).

Treatment was generally well tolerated in the 3 treatment groups. In total only 6 patients (5%) had grade 3 or 4 toxicities, 4 in the 5FU-RT group, one in the XRT group and one in the chemotherapy-only group. Diarrhea was the most common grade 3 or 4 adverse effect. One

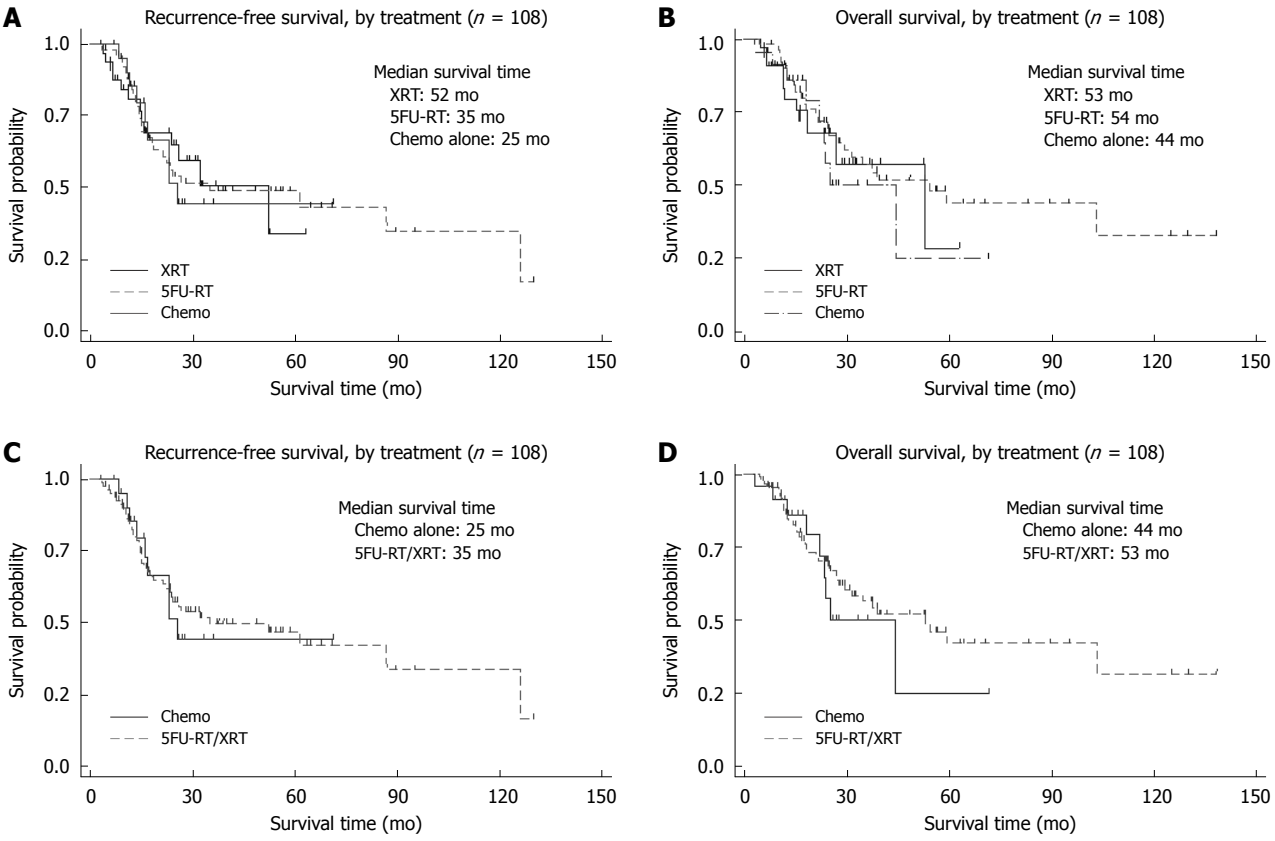

Figure 1 Kaplan-Meier estimates. A: Recurrence-free survival of capecitabine + radiation (XRT), 5-fluorouracil + radiation (5FU-RT) and chemotherapy alone; B: Overall Survival of XRT, 5FU-RT and chemotherapy alone; C: Recurrence-free survival of RT-containing regimen *vs* chemotherapy alone; D: Overall survival of RT-containing regimen *vs* chemotherapy alone.

Table 2 Recurrence-free survival and overall survival analysis

Variable	Categories/units	*n*	HR (95% CI)	*P*-value
Recurrence-free survival				
Treatment	XRT	108	1	
	5FU-RT		1.00 (0.508-1.959)	1.0
	Chemo only		1.05 (0.448-2.462)	0.9
Treatment	XRT	108	1	
	5FU-RT		0.97[1] (0.477-1.966)	0.9
	Chemo only		1.03[1] (0.433-2.444)	0.9
Overall survival				
Treatment	XRT	108	1	
	5FU-RT		0.83 (0.411-1.681)	0.6
	Chemo only		1.13 (0.474-2.677)	0.8
Treatment	XRT	108	1	
	5FU-RT		0.89[1] (0.428-1.856)	0.8
	Chemo only		1.19[1] (0.494-2.882)	0.7

[1]Age-adjusted hazard ratio (HR). XRT: Capecitabine + radiation; 5FU-RT: 5-fluorouracil + radiation.

patient in the XRT group had acute myocardial infarction during the therapy period. There was one death from non-neutropenic sepsis during the adjuvant treatment in the 5FU-RT group.

DISCUSSION

Our study showed that XRT was as effective as 5FU-RT and fluoropyrimidine-based chemotherapy when given as adjuvant treatment for locally advanced gastric cancer. XRT had comparable recurrence-free and overall survival with the other 2 adjuvant regimens.

The Intergroup-0116 study has established the role of adjuvant chemoradiation for resected locally advanced gastric cancer in the United States. Compared to surgery alone, the addition of chemoradiation after resection leads to an increased local control (30 mo *vs* 19 mo, $P = 0.001$) and better median overall survival (36 mo *vs* 27 mo, $P = 0.005$)[7]. In our study, the median overall survival for patients receiving XRT or 5FU-RT was 53 and 54 mo, respectively. This outcome when compared to the Intergroup trial is encouraging as 66% of patients in our study population had T3 or T4 disease and 86% had lymph node-positive disease, similar to the study population in the Intergroup study (68% and 85%, respectively). This difference could be explained by the differences in surgical techniques. All except 2 of our patients underwent D2 gastrectomies compared to only 10% in the Intergroup study. This may suggest that the adjuvant treatment could be a measure to compensate for inadequate surgical treatment. Nevertheless, the extent of lymph node dissection remains an ongoing debate with trials from the Dutch Gastric Cancer Group[8] and Medical Research Council[9] showing a lack of survival benefit of D2 over D1 lymph node dissection. However, the role of adjuvant chemoradiation in D2-resected gastric cancer had been studied in a Korean prospective non-randomized trial involving 544 patients receiving postoperative 5FU-

Table 3 Recurrence-free survival and overall survival analysis

Variable	Categories/units	Chemo alone		5FU-RT		XRT	
		HR (95% CI)	*P*-value[1]	HR (95% CI)	*P*-value[1]	HR (95% CI)	*P*-value[1]
Recurrence-free survival							
ECOG	0	1	0.9	1	1.0	E/N = 0/1	0.5
	1	0.92 (0.115-7.422)		1.01 (0.238-4.326)		NE	
Gender	Male	1	0.9	1	0.2	1	0.5
	Female	0.91 (0.244-3.419)		1.58 (0.727-3.436)		0.65 (0.198-2.163)	
Surgical margins	Positive	1	0.5	1	< 0.001	1	0.03
	Negative	0.51 (0.059-4.351)		0.16 (0.070-0.384)		0.27 (0.081-0.918)	
Stage	1 or 2	1	0.1	1	0.2	1	0.06
	3 or 4	2.92 (0.724-11.773)		1.86 (0.749-4.633)		3.87 (0.853-17.586)	
Grade of tumor	1, 2	1	0.3	1	0.09	1	0.3
	3	2.17 (0.444-10.636)		2.44 (0.836-7.099)		1.94 (0.515-7.288)	
Overall survival							
ECOG	0	1	0.8	1	0.9	E/N = 0/1	0.4
	1	0.78 (0.096-6.366)		0.95 (0.220-4.069)		NE	
Gender	Male	1	1.0	1	0.2	8/22	0.7
	Female	1.02 (0.253-4.148)		1.63 (0.740-3.598)		0.78 (0.234-2.597)	
Surgical margins	Positive	1	0.004	1	< 0.001	4/4	0.02
	Negative	0.10 (0.013-0.700)		0.26 (0.113-0.603)		0.25 (0.071-0.901)	
Stage	1 or 2	1	0.2	1	0.1	2/12	0.06
	3 or 4	2.59 (0.646-10.412)		2.08 (0.778-5.557)		3.80 (0.829-17.397)	
Grade of tumor	1, 2	1	0.4	1	0.06	3/12	0.4
	3	2.04 (0.418-9.944)		3.04 (0.903-10.239)		1.83 (0.482-6.980)	

[1]*P* -values were calculated using the Log-rank test. 5FU-RT: 5-fluorouracil + radiation; XRT: Capecitabine + radiation; ECOG: Eastern Cooperative Oncology Group status; E/N: Number of events/number of patients in the group; NE: Not estimable; HR: Hazard ratio.

Table 4 Subgroup analysis for overall and recurrence-free survival

Subgroup	Variable	Categories/units	*n*	Overall survival		Recurrence-free survival	
				HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Surgical margins = positive	Treatment	Chemo alone	21	1		1	
		5FU-RT/XRT		0.47 (0.100–2.191)	0.3	1.58 (0.206-12.154)	0.7
Stage T3/4	Treatment	Chemo alone	66	1		1	
		5FU-RT/XRT		0.65 (0.294–1.454)	0.3	0.78 (0.352-1.709)	0.5
Node positive	Treatment	Chemo alone	94	1		1	
		5FU-RT/XRT		0.92 (0.404–2.081)	0.8	1.10 (0.489-2.482)	0.8

5FU-RT: 5-fluorouracil + radiation; XRT: Capecitabine + radiation; HR: Hazard ratio.

radiation. This trial demonstrated significantly longer overall survival in the chemoradiation group compared to the surgery alone group (95.3 mo *vs* 62.6 mo, $P = 0.02$)[10]. Hence, there appears to be a role of adjuvant concurrent chemoradiation even in the setting of optimal surgical resection of gastric cancer.

Continuous infusion 5-FU was preferred over 5-FU bolus infusion in most of the gastrointestinal malignancies, especially colorectal cancer, and was also found to be more effective than the bolus 5-FU[11,12]. Continuous 5-FU infusion with radiation was used extensively in the neoadjuvant and adjuvant treatment of rectal cancer[13,14]. Oral capecitabine, in the metastatic and adjuvant setting, has been shown to be as effective as continuous 5-FU in gastrointestinal malignancies[2,12,15,16]. The ease of oral administration of capecitabine compared with the continuous infusion of 5-FU, which requires the placement of a central venous catheter, makes the use of capecitabine concurrent with radiotherapy an attractive option. Our results, albeit retrospective, showed an equivalent survival between XRT and 5FU-RT. Hence, XRT could be a reasonable alternative to 5FU-RT as adjuvant treatment in resected stomach cancer. Several phase Ⅰ / Ⅱ studies have already explored the addition of capecitabine with concurrent radiation[4,5] in adjuvant stomach cancer and shown it to be safe and tolerable.

Our analysis has shown that there was no survival difference between those who had radiation as part of their adjuvant therapy compared to those who did not. Even in patients with a positive surgical margin, the addition of radiation did not appear to significantly improve survival compared to adjuvant chemotherapy alone. The role of adjuvant chemotherapy had been studied in many phase Ⅲ Western trials but the results were inconsistent in showing a survival benefit of adjuvant chemotherapy over surgery alone[17-20]. Meta-analyses of these trials suggest a potential absolute increase in 5-year survival of 2% to 4% with adjuvant chemotherapy in resected gastric

cancer[21,22]. The Asian adjuvant trials had demonstrated more favorable results with a recent Japanese study on adjuvant S1 in resected stage Ⅱ or Ⅲ gastric cancer showing a significantly higher 3-year overall survival rate in the S1 group compared to the observation arm (80.1% *vs* 70.1%, $P = 0.003$)[23]. Hence, fluoropyrimidine-based chemotherapy may have a role in adjuvant treatment. However, there is currently no phase Ⅲ trial that compared adjuvant chemoradiation and chemotherapy alone. Our study has shown that there is no survival difference between adjuvant chemoradiation and chemotherapy, suggesting that adjuvant chemotherapy alone may be a reasonable option of adjuvant therapy in resected gastric cancer. A study involving capecitabine alone for adjuvant therapy in gastric cancer will be an interesting follow-up study.

In conclusion, XRT as an adjuvant therapy in resected gastric cancer can achieve similar outcomes to that of 5FU-RT or chemotherapy. The result from our hypothesis-generating study provides the basis for a further prospective study in evaluating the role of radiation with concurrent capecitabine as adjuvant therapy in resected gastric cancers.

COMMENTS

Background

Gastric cancer is a major cause of cancer deaths in the world. The outcome of large gastric tumors and those with lymph node involvement remains poor after surgical resection. The optimal adjuvant therapy after surgical resection remains to be determined.

Research frontiers

The most common strategies in the adjuvant treatment of gastric cancers include fluoropyrimidine-based chemotherapy with or without radiation. The introduction of capecitabine has largely replaced continuous-infusion 5-fluorouracil (5-FU) owing to its ease of administration. However, its efficacy is not proven in randomized phase Ⅲ trials involving gastric cancers. In this retrospective review study, the authors examined the role of capecitabine with radiation and compared its efficacy to the 5-FU with radiation regimen and fluoropyrimidine-based chemotherapy alone.

Innovations and breakthroughs

This study showed that capecitabine with concurrent radiation was as effective as 5-FU with radiation or fluoropyrimidine-based chemotherapy alone without radiation when given as adjuvant treatment for locally advanced gastric cancer.

Applications

This hypothesis-generating study will provide the platform for a larger randomized study to be conducted using capecitabine as one of the study regimens in adjuvant gastric cancer trials.

Terminology

Capecitabine and 5-FU are fluoropyrimidine-based chemotherapy commonly used in the treatment of gastrointestinal cancers.

Peer review

This is a retrospective clinical study that looked at the effects of chemoradiation therapy by using capecitabine in patients undergoing gastric surgery for gastric cancer. The results are well presented and the discussion is well organized. The conclusions are supported by the data and the tables contain appropriate information.

REFERENCES

1 **Catalano V**, Labianca R, Beretta GD, Gatta G, de Braud F, Van Cutsem E. Gastric cancer. *Crit Rev Oncol Hematol* 2009; **71**: 127-164

2 **Cunningham D**, Starling N, Rao S, Iveson T, Nicolson M, Coxon F, Middleton G, Daniel F, Oates J, Norman AR. Capecitabine and oxaliplatin for advanced esophagogastric cancer. *N Engl J Med* 2008; **358**: 36-46

3 **Kang YK**, Kang WK, Shin DB, Chen J, Xiong J, Wang J, Lichinitser M, Guan Z, Khasanov R, Zheng L, Philco-Salas M, Suarez T, Santamaria J, Forster G, McCloud PI. Capecitabine/cisplatin versus 5-fluorouracil/cisplatin as first-line therapy in patients with advanced gastric cancer: a randomised phase III noninferiority trial. *Ann Oncol* 2009; **20**: 666-673

4 **Jansen EP**, Boot H, Saunders MP, Crosby TD, Dubbelman R, Bartelink H, Verheij M, Cats A. A phase I-II study of postoperative capecitabine-based chemoradiotherapy in gastric cancer. *Int J Radiat Oncol Biol Phys* 2007; **69**: 1424-1428

5 **Lee HS**, Choi Y, Hur WJ, Kim HJ, Kwon HC, Kim SH, Kim JS, Lee JH, Jung GJ, Kim MC. Pilot study of postoperative adjuvant chemoradiation for advanced gastric cancer: adjuvant 5-FU/cisplatin and chemoradiation with capecitabine. *World J Gastroenterol* 2006; **12**: 603-607

6 **Kim DY**, Jung KH, Kim TH, Kim DW, Chang HJ, Jeong JY, Kim YH, Son SH, Yun T, Hong CW, Sohn DK, Lim SB, Choi HS, Jeong SY, Park JG. Comparison of 5-fluorouracil/leucovorin and capecitabine in preoperative chemoradiotherapy for locally advanced rectal cancer. *Int J Radiat Oncol Biol Phys* 2007; **67**: 378-384

7 **Macdonald JS**, Smalley SR, Benedetti J, Hundahl SA, Estes NC, Stemmermann GN, Haller DG, Ajani JA, Gunderson LL, Jessup JM, Martenson JA. Chemoradiotherapy after surgery compared with surgery alone for adenocarcinoma of the stomach or gastroesophageal junction. *N Engl J Med* 2001; **345**: 725-730

8 **Bonenkamp JJ**, Hermans J, Sasako M, van de Velde CJ, Welvaart K, Songun I, Meyer S, Plukker JT, Van Elk P, Obertop H, Gouma DJ, van Lanschot JJ, Taat CW, de Graaf PW, von Meyenfeldt MF, Tilanus H. Extended lymph-node dissection for gastric cancer. *N Engl J Med* 1999; **340**: 908-914

9 **Hartgrink HH**, van de Velde CJ, Putter H, Bonenkamp JJ, Klein Kranenbarg E, Songun I, Welvaart K, van Krieken JH, Meijer S, Plukker JT, van Elk PJ, Obertop H, Gouma DJ, van Lanschot JJ, Taat CW, de Graaf PW, von Meyenfeldt MF, Tilanus H, Sasako M. Extended lymph node dissection for gastric cancer: who may benefit? Final results of the randomized Dutch gastric cancer group trial. *J Clin Oncol* 2004; **22**: 2069-2077

10 **Kim S**, Lim DH, Lee J, Kang WK, MacDonald JS, Park CH, Park SH, Lee SH, Kim K, Park JO, Kim WS, Jung CW, Park YS, Im YH, Sohn TS, Noh JH, Heo JS, Kim YI, Park CK, Park K. An observational study suggesting clinical benefit for adjuvant postoperative chemoradiation in a population of over 500 cases after gastric resection with D2 nodal dissection for adenocarcinoma of the stomach. *Int J Radiat Oncol Biol Phys* 2005; **63**: 1279-1285

11 Efficacy of intravenous continuous infusion of fluorouracil compared with bolus administration in advanced colorectal cancer. Meta-analysis Group In Cancer. *J Clin Oncol* 1998; **16**: 301-308

12 **Meyerhardt JA**, Mayer RJ. Systemic therapy for colorectal cancer. *N Engl J Med* 2005; **352**: 476-487

13 **Sauer R**, Becker H, Hohenberger W, Rödel C, Wittekind C, Fietkau R, Martus P, Tschmelitsch J, Hager E, Hess CF, Karstens JH, Liersch T, Schmidberger H, Raab R. Preoperative versus postoperative chemoradiotherapy for rectal cancer. *N Engl J Med* 2004; **351**: 1731-1740

14 **Bosset JF**, Collette L, Calais G, Mineur L, Maingon P, Radosevic-Jelic L, Daban A, Bardet E, Beny A, Ollier JC. Chemotherapy with preoperative radiotherapy in rectal cancer. *N Engl J Med* 2006; **355**: 1114-1123

15 **Van Cutsem E**, Twelves C, Cassidy J, Allman D, Bajetta E, Boyer M, Bugat R, Findlay M, Frings S, Jahn M, McKendrick J, Osterwalder B, Perez-Manga G, Rosso R, Rougier P, Schmiegel WH, Seitz JF, Thompson P, Vieitez JM, Weitzel C, Harper P. Oral capecitabine compared with intravenous fluorouracil plus leucovorin in patients with metastatic colorectal

cancer: results of a large phase III study. *J Clin Oncol* 2001; **19**: 4097-4106

16 **Twelves C**, Wong A, Nowacki MP, Abt M, Burris H 3rd, Carrato A, Cassidy J, Cervantes A, Fagerberg J, Georgoulias V, Husseini F, Jodrell D, Koralewski P, Kröning H, Maroun J, Marschner N, McKendrick J, Pawlicki M, Rosso R, Schüller J, Seitz JF, Stabuc B, Tujakowski J, Van Hazel G, Zaluski J, Scheithauer W. Capecitabine as adjuvant treatment for stage III colon cancer. *N Engl J Med* 2005; **352**: 2696-2704

17 **Nitti D**, Wils J, Dos Santos JG, Fountzilas G, Conte PF, Sava C, Tres A, Coombes RC, Crivellari D, Marchet A, Sanchez E, Bliss JM, Homewood J, Couvreur ML, Hall E, Baron B, Woods E, Emson M, Van Cutsem E, Lise M. Randomized phase III trials of adjuvant FAMTX or FEMTX compared with surgery alone in resected gastric cancer. A combined analysis of the EORTC GI Group and the ICCG. *Ann Oncol* 2006; **17**: 262-269

18 **Bajetta E**, Buzzoni R, Mariani L, Beretta E, Bozzetti F, Bordogna G, Aitini E, Fava S, Schieppati G, Pinotti G, Visini M, Ianniello G, Di BM. Adjuvant chemotherapy in gastric cancer: 5-year results of a randomised study by the Italian Trials in Medical Oncology (ITMO) Group. *Ann Oncol* 2002; **13**: 299-307

19 **Di Costanzo F**, Gasperoni S, Manzione L, Bisagni G, Labianca R, Bravi S, Cortesi E, Carlini P, Bracci R, Tomao S, Messerini L, Arcangeli A, Torri V, Bilancia D, Floriani I, Tonato M, Dinota A, Strafiuso G, Corgna E, Porrozzi S, Boni C, Rondini E, Giunta A, Monzio Compagnoni B, Biagioni F, Cesari M, Fornarini G, Nelli F, Carboni M, Cognetti F, Enzo MR, Piga A, Romiti A, Olivetti A, Masoni L, De Stefanis M, Dalla Mola A, Camera S, Recchia F, De Filippis S, Scipioni L, Zironi S, Luppi G, Italia M, Banducci S, Pisani Leretti A, Massidda B, Ionta MT, Nicolosi A, Canaletti R, Biscottini B, Grigniani F, Di Costanzo F, Rovei R, Croce E, Carroccio R, Gilli G, Cavalli C, Olgiati A, Pandolfi U, Rossetti R, Natalini G, Foa P, Oldani S, Bruno L, Cascinu S, Catalano G, Catalano V, Lungarotti F, Farris A, Sarobba MG, Trignano M, Muscogiuri A, Francavilla F, Figoli F, Leoni M, Papiani G, Orselli G, Antimi M, Bellini V, Cabassi A, Contu A, Pazzola A, Frignano M, Lastraioli E, Saggese M, Bianchini D, Antonuzzo L, Mela M, Camisa R. Adjuvant chemotherapy in completely resected gastric cancer: a randomized phase III trial conducted by GOIRC. *J Natl Cancer Inst* 2008; **100**: 388-398

20 **Bouché O**, Ychou M, Burtin P, Bedenne L, Ducreux M, Lebreton G, Baulieux J, Nordlinger B, Martin C, Seitz JF, Tigaud JM, Echinard E, Stremsdoerfer N, Milan C, Rougier P. Adjuvant chemotherapy with 5-fluorouracil and cisplatin compared with surgery alone for gastric cancer: 7-year results of the FFCD randomized phase III trial (8801). *Ann Oncol* 2005; **16**: 1488-1497

21 **Earle CC**, Maroun JA. Adjuvant chemotherapy after curative resection for gastric cancer in non-Asian patients: revisiting a meta-analysis of randomised trials. *Eur J Cancer* 1999; **35**: 1059-1064

22 **Mari E**, Floriani I, Tinazzi A, Buda A, Belfiglio M, Valentini M, Cascinu S, Barni S, Labianca R, Torri V. Efficacy of adjuvant chemotherapy after curative resection for gastric cancer: a meta-analysis of published randomised trials. A study of the GISCAD (Gruppo Italiano per lo Studio dei Carcinomi dell'Apparato Digerente). *Ann Oncol* 2000; **11**: 837-843

23 **Sakuramoto S**, Sasako M, Yamaguchi T, Kinoshita T, Fujii M, Nashimoto A, Furukawa H, Nakajima T, Ohashi Y, Imamura H, Higashino M, Yamamura Y, Kurita A, Arai K. Adjuvant chemotherapy for gastric cancer with S-1, an oral fluoropyrimidine. *N Engl J Med* 2007; **357**: 1810-1820

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BRIEF ARTICLE

Stent-grafts placement for treatment of massive hemorrhage from ruptured hepatic artery after pancreaticoduodenectomy

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Abstract

AIM: To present a series of cases with life-threatening hemorrhage from ruptured hepatic artery pseudoaneurysm after pancreaticoduodenectomy (PD) treated with placement of stent-grafts.

METHODS: Massive hemorrhage from ruptured hepatic artery pseudoaneurysm after PD in 9 patients (6 men, 3 women) at the age of 23-75 years (mean 48 years), were treated with placement of percutaneous endovascular balloon-expandable coronary stent-grafts. All patients were not suitable for embolization because of a non-patent portal vein. One or more stent-grafts, ranging 3-6 mm in diameter and 16-55 mm in length, were placed to exclude ruptured pseudoaneurysm. Follow-up data, including clinical condition, liver function tests, and Doppler ultrasound examination, were recorded at the outpatient clinic.

RESULTS: Immediate technical success was achieved in all the 9 patients. All stent-grafts were deployed in the intended position for immediate cessation of bleeding and preservation of satisfactory hepatic arterial blood flow. No significant procedure-related complications occurred. Recurrent bleeding occurred in 2 patients at 16 and 24 h, respectively, after placement of stent-grafts and treated with surgical revision. One patient died of sepsis 12 d after the interventional procedure. The remaining 6 patients were survived when they were discharged. The mean follow-up time was 10.5 mo (range 4-16 mo). No patient had recurrent bleeding after discharge. Doppler ultrasound examination verified the patency of hepatic artery and stent-grafts during the follow-up.

CONCLUSION: Placement of stent-grafts is an effective and safe procedure for acute life-threatening hemorrhage from ruptured hepatic artery pseudoaneurysm.

Key words: Pancreaticoduodenectomy; Hemorrhage; Hepatic artery; Pseudoaneurysm; Stent-graft

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INTRODUCTION

Delayed hepatic arterial hemorrhage after pancreaticodu-

odenectomy (PD) is not a common, but a potentially fatal complication[1-4]. Massive hepatic arterial bleeding occurs as a result of inflammatory vascular erosion related to pancreatic juice or bile leaking from an insufficient anastomosis and/or due to local infection. Treatment options include re-operation or endovascular catheter techniques such as coil embolization of the bleeding vessels. Surgical exploration and identification of the bleeding vessel may be difficult in acute situations and hazardous because of adhesions and surrounding postsurgical tissue friability[5,6]. Embolization of the bleeding artery has an immediate impact on patient survival but often excludes the distal circulation, which may be a risk factor for hepatic ischemia and even fatal hepatic necrosis, particularly in patients with portal vein stenosis or thrombosis[3,7-9].

Recently the use of stent-grafts to exclude aneurysms in splenic and other visceral arteries has been presented in single case reports[10-15]. We report our experience in 9 patients with delayed massive hemorrhage from ruptured hepatic artery pseudoaneurysm after PD, demonstrating the potential of interventional radiology in initial treatment of hemorrhage, instead of re-operation and coil embolization, using transluminal stent graft placement for emergency vessel repair retaining patency of hepatic artery.

MATERIALS AND METHODS

Patients

From March 2003 to December 2009, 9 patients (6 men, 3 women) at the age of 23-75 years (mean 48 years), referred to our department for delayed massive hemorrhage from ruptured hepatic artery pseudoaneurysm occurring 6 or more days after PD, were treated with percutaneous endovascular placement of balloon-expandable stent-grafts. All medical records, radiological reports, and images of the patients were retrospectively reviewed. Delayed massive hemorrhage occurring 5 or more days after PD in patients with stable hemodynamics was defined as a potentially life-threatening bleeding leading to hemorrhagic shock needing blood transfusions as previously described[2,3].

The indications for PD are listed in Table 1. Classic resection procedures were performed in all the 9 patients, including Whipple procedure (n = 6) and pylorus preserving pancreatoduodenectomy (n = 3). Pancreatic fistula (PF) after PD, diagnosed by routine assay of drainage fluid amylase levels, was present in 7 patients with local infection. PF was defined as drain output of any measurable volume of fluid on or after postoperative day 3 with amylase content 3 times greater than that of serum amylase activity[4]. Embolization of splenic artery was performed in 2 patients (Patients No. 1 and No. 2) due to bleeding from the splenic artery.

All the 9 patients presented with an unstable clinical condition with their heart rate higher than 90 beats per min, blood pressure lower than 80/40 mmHg, hemoglobin lower than 6.5 g/dL (reference range 13-18 g/dL), hematocrit lower than 40% (reference range 40%-54%), and blood transfusion greater than 5 U. Angiography revealed active hemorrhage from the hepatic artery pseudoaneurysm, including bleeding from the abdominal drain (n = 4), gastrointestinal tract (n = 2), or both (n = 3). Bleeding occurred in 7 patients (78%) before they were discharged and in 2 patients (Patients No. 5 and No. 7) after they were discharged following an uneventful postoperative course. The mean time between PD and onset of massive bleeding was 15.3 d (range 6-38 d).

All patients received an average blood transfusion of 10.6 U (9-12 U) before the interventional procedure. Of the 9 patients, 5 needed an average of 1520 mL (1200-1800 mL) fresh frozen plasma and 4 required intubation.

Emergency endoscopy performed in 3 patients showed mixed old and fresh blood in gastric lumen but no bleeding site. Computed tomography (CT) and ultrasonography were not performed because of the emergency situation.

Informed consent was obtained from the patients or their guardians before the interventional procedure.

Indication for stent graft placement

Instead of coil embolization, stent-graft was placed to exclude hepatic pseudoaneurysm because the patients had an obstructed portal vein prior to massive pseudoaneurysm bleeding, including thrombosis of the portal vein in 5 patients, tumor infiltration of the portal vein in 3 patients, and ligation of the portal vein during the surgical procedure in 1 patient. In patients with a non-patent portal vein, embolization of the common or proper hepatic artery was a high risk factor for hepatic ischemia and even fatal hepatic necrosis as previously described[9,16].

Endovascular techniques

After restoration of hemodynamic stability by aggressive resuscitation with intravenous fluids and administration of blood products, the patients underwent emergency abdominal angiography with standard Seldinger technique. All procedures were performed under local anesthesia (2% lidocaine). The patients were monitored by electrocardiogram and blood pressure measurements.

A pigtail catheter (4 Fr, Cordis, the Netherlands) was inserted into the abdominal aorta at level of the 12th thoracic vertebra, and an abdominal aortography was performed with digital subtraction angiography technique. The celiac trunk, hepatic artery, splenic artery, and superior mesenteric artery were selectively catheterized using a 4 Fr Cobra catheter (Cordis). Both arterial and portal venous phases were assessed, in order to detect the bleeding site and exclude the portal vein thrombosis.

Diameter of the affected artery at the located bleeding site was measured. A 0.35-inch guide-wire (Terumo Co., Tokyo, Japan) was passed into a distal branch of the right or left hepatic artery through the 4 Fr Cobra catheter under fluoroscopic guidance. The femoral 4 Fr introducer was replaced with an 8 Fr introducer with a 60 cm long vascular sheath (Arrow, Arrow International, USA).

A stent-graft (Jostent, Graftmaster, Coronary stent graft, Germany) was advanced into the distal extravasation site through the 8 Fr Arrow sheath over a 0.014 inch guide-wire (Table 2). The sheath was then pulled back to

Table 1 Clinical data about the patients with bleeding hepatic artery pseudoaneurysm following pancreaticoduodenectomy treated with stent grafts placement

Patient No.	Age (yr)/sex	Indication for surgery	PF	Bleeding at POD	Initial presentation of bleeding	Transfusion of units of blood	Stent-graft
1	75/F	Carcinoma of pancreatic head	Yes	14	Abdominal drain	9, 9 FFP	Jostent 4 mm × 19 mm × 2 pieces
2	23/F	Pancreatic trauma	Yes	9	Abdominal drain	12	Jostent 4 mm × 19 mm × 2 pieces
3	42/M	Distal common bile cholangiocarcinoma	Yes	15	Abdominal drain	11, 6 FFP	Jostent 4 mm × 19 mm × 3 pieces
4	56/M	Carcinoma of pancreatic head	Yes	7	Abdominal drain and hematemesis	11, 7 FFP	Jostent 3.5 mm × 19 mm × 1 piece 4 mm × 19 mm × 1 piece
5	62/M	Carcinoma of pancreatic head	No	35	Hematemesis and melena	9	Jostent 4 mm × 19 mm × 1 piece 4 mm × 16 mm × 1 piece
6	67/M	Pancreatic carcinoma	Yes	6	Surgical drainage, nasogastric tube	11	Jostent 6 mm × 55 mm × 1 piece
7	53/M	Periampullary cancer	No	38	Hematemesis and melena	9	Jostent 4 mm × 19 mm × 3 pieces
8	68/M	Pancreatic carcinoma	Yes	8	Abdominal drain	11, 9 FFP	Jostent 3.5 mm × 19 mm × 1 piece 4 mm × 16 mm × 1 piece
9	50/F	Carcinoma of pancreatic head	Yes	6	Surgical drainage, nasogastric tube	12, 7 FFP	Jostent 4 mm × 19 mm × 2 pieces

PD: Pancreaticoduodenectomy; POD: Postoperative day; FFP: Fresh frozen plasma; PF: Pancreatic fistula.

Table 2 Outcome of the patients enrolled in this study after placement of stent grafts

Patient No.	Immediate technical success	Intensive care unit/ length of stay	Clinical outcome	Length of hospital stay (d)	Follow-up
1	Yes	Yes/19 d	Bleeding stopped, no further hemorrhage	48	4 mo exitus, AMI
2	Yes	Yes/7 d	Bleeding stopped, no further bleeding	28	16 mo, clinical and laboratory findings normal
3	Yes	Yes/10 d	Bleeding stopped, no further bleeding	38	10 mo exitus, underlying malignancy
4	Yes	Yes/15 d	Recurrent bleeding 16 h later, underwent surgical revision	32	2 d exitus, uncontrolled bleeding
5	Yes	No	Cessation of bleeding, no further hemorrhage	11	14 mo exitus, underlying malignancy
6	Yes	Yes/8 d	Bleeding stopped, no further hemorrhage	36	8 mo exitus, underlying malignancy
7	Yes	No	Bleeding stopped, no further hemorrhage	12	11 mo, clinical and laboratory findings normal
8	Yes	Yes/21 d	Recurrent bleeding 24 h later, underwent surgical revision	40	3 d exitus, multiorgan failure
9	Yes	Yes/24 d	Bleeding stopped, no further hemorrhage	43	12 d exitus, abdominal sepsis

AMI: Acute myocardial infarction.

the proximal extravasation site to allow initial exposure of the stent-graft. The stent-graft was then deployed by inflating the balloon to a pressure of 8 atmospheres. A 6/20-mm balloon catheter (Abbott Lab, IL) was then used to post-dilate the stent-graft. If placement of one stent-graft failed to completely exclude the pseudoaneurysm, a second or even a third stent-graft was placed in a coaxial overlapping manner. Control angiography was repeated to confirm the exclusion of pseudoaneurysm, no sign of contrast medium extravasation, and patency of the hepatic artery.

Post-procedural management

All individuals were given antibiotics to prevent infection with aerobic and non-aerobic bacteria but no anticoagulation or anti-platelet drugs immediately after the procedure due to the emergent hemorrhagic conditions.

Six patients were survived when they were discharged and aspirin (100 mg/d) was given lifelong after discharge. Follow-up data, including clinical condition and laboratory (liver function test) findings, were recorded at the outpatient clinic. Doppler ultrasound studies were performed every day for 1 wk after placement of stent-grafts, then every 2-3 d, followed by every month on an outpatient basis.

Outcome parameters

Technical success was defined as the successful deploy-

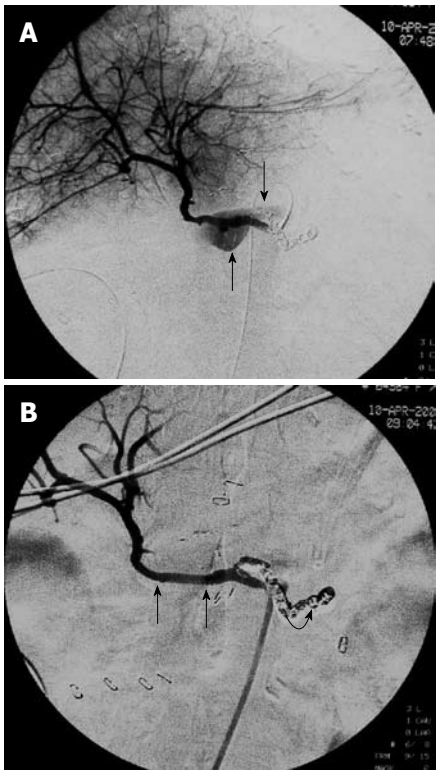

Figure 1 A 75-year-old female with pancreatic head malignancy underwent a pylorus-preserving pancreaticoduodenectomy (Case 1). A: Selective angiography of the common hepatic artery shows a large ruptured hepatic artery pseudoaneurysm with extravasation of contrast medium (arrows); B: Angiography after two stent-grafts placement demonstrates exclusion of the aneurysm and preserved hepatic artery blood flow through the stent-grafts (arrows). Note the splenic artery was embolized 4 d ago due to bleeding from the splenic artery (curved arrow).

ment of stent-graft within the intended artery, exclusion of pseudoaneurysm without evidence for contrast extravasation, cessation of immediate hemorrhage, and preservation of hepatic arterial flow. Clinical success was defined as the disappearance of signs or symptoms and improvement in laboratory findings. Re-bleeding from the same arterial focus after treatment was defined as clinical failure.

RESULTS

Immediate technical success

The selective angiography of celiac axis demonstrated active bleeding (extravasation of contrast agent) from the ruptured hepatic artery pseudoaneurysm in all patients (Figure 1A). A non-patent main portal vein was observed at the delayed phase in all patients.

Stent-graft placement was technically successful in all patients with the bleeding pseudoaneurysm completely excluded and stable hemodynamically achieved immediately (Figure 1B). The control angiography demonstrated patency of the hepatic artery in all individuals (Figure 2A and B). Each patient needed one or more stent-grafts to exclude his or her ruptured hepatic pseudoaneurysm. A total of 19 stent-grafts were implanted in 9 patients (Table 1).

The median time of interventional procedure, including diagnostic angiography, was 65 min (range 35-100 min).

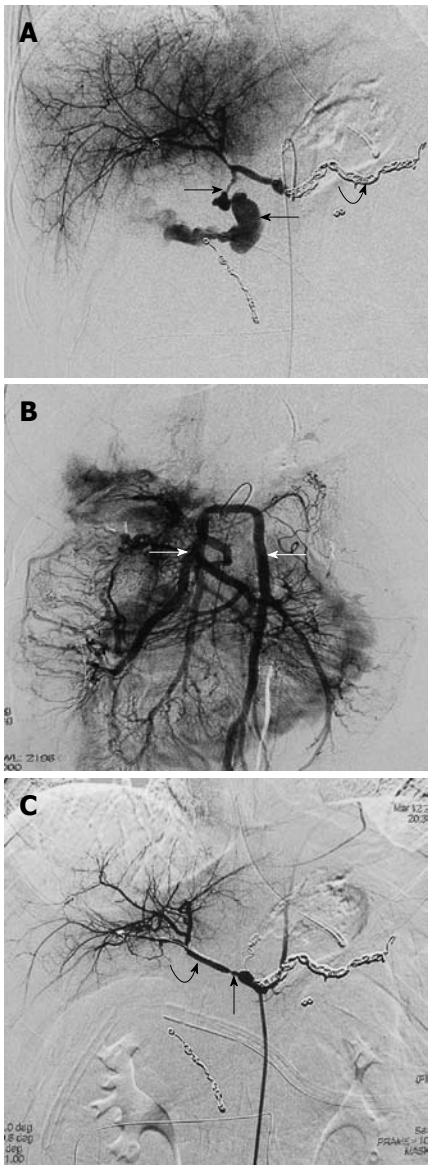

Figure 2 A 23-year-old female with pancreatic trauma underwent a pylorus-preserving pancreaticoduodenectomy (Case 2). A: Emergent selective angiography of the celiac trunk demonstrates massive extravasation of contrast medium into the abdominal cavity from the ruptured hepatic artery pseudoaneurysm (arrows). Note the splenic artery was embolized 6 d ago due to bleeding from the splenic artery (curved arrow); B: Digital substraction superior mesenteric artery angiogram with delayed phase demonstrates no visualization of the portal vein, and remarkable pooling of contrast medium at the branches of the SMV (arrows); C: Angiography after two pieces of stent-grafts placement shows exclusion of the bleeding hepatic pseudoaneurysm and good hepatic arterial blood flow through the stent-grafts (curved arrow). Note the spasm of the proximal hepatic artery (arrow).

No vascular adverse event occurred during the procedure. Control angiography showed vasospasm of the proximal and distal hepatic artery during and immediately after the procedure as expected because of the intra-arterial guide wire manipulation (Figure 2C), which did not affect the outcome of intervention. No major complication of the procedure was observed.

Clinical outcome

Seven patients were transferred to intensive care unit after

placement of stent-grafts with a mean stay time of 13.7 d (7-24 d). Four patients required intubation.

Hemodynamically stabilization was achieved in 7 of the 9 patients after the procedure without evidence of further bleeding (Table 2). Laboratory findings (liver function tests) were within the normal range 1, 3 and 7 d after the procedures and at the time when the patients were discharged.

Two patients (Patients No. 4 and No. 8) with clinical failure underwent surgical revision. In patient No. 4, the stent-graft was successfully placed, but his hemoglobin level dropped again 16 h after the procedure due to bleeding from the same artery probably due to the dislodgment of stent-grafts. This patient underwent surgical revision and died of uncontrolled bleeding 2 d after the procedure. In patient No. 8, recurrent bleeding occurred 24 h after the placement of stent-grafts due to bleeding from the same artery. He underwent surgical revision with haemostasis achieved. The patient died of multiorgan failure 3 d after the procedure.

Of the 7 patients with their bleeding completely controlled, one (Patient No. 9) died of intra-abdominal sepsis 12 d after the interventional procedure with no active bleeding until her death. Four patients were subjected to CT-guided interventional placement of additional drains close to the pancreaticojejunostomy due to considerable fluid collections. Two patients underwent re-operation for persistent abscess and PF.

Follow-up

Six patients were survived when they were discharged (Table 2). Their mean hospital stay time was 24.5 d (range 11-48 d). The mean follow-up time was 10.5 mo (range 4-16 mo). No further bleeding was seen during the follow-up. Clinical and laboratory follow-up findings were unremarkable. Doppler ultrasound examination verified the patency of hepatic artery and stent-grafts during the follow-up.

DISCUSSION

It was reported that approximately two thirds of delayed arterial hemorrhage cases after PD have an underlying collection or anastomotic leak, with pseudoaneurysm formation[1,2,4,5,9]. In our study, delayed hepatic arterial hemorrhage occurred in 7 of the 9 patients due to the local complication (PF).

Transcatheter arterial embolization (TAE) has been advocated as the first-line treatment modality for late-onset bleeding after PD[17-20], with a success rate of 83%-100% and a mortality of 0%-20%. In fact, the liver can tolerate embolization of the main hepatic artery without major consequences, since it has a dual blood supply from the portal and arterial circulations. Collateral arterial blood flow to the liver can also be expected. However, the number of collaterals is less than that of normal blood vessels after PD because of lymphadenectomy and skeletonization of the vasculature[3]. Embolization of the hepatic artery may result in liver failure and necrosis as well as intrahepatic abscesses[5,21,22].

In this study, all patients had an obstructed portal vein. Embolization of the hepatic artery itself, proximal and distal to the bleeding pseudoaneurysm, may influence the liver perfusion with an undesirable effect[9,16]. Thus, for preserving hepatic arterial blood flow, implantation of stent-grafts (covered stent) may be better than TAE[10-12].

In recent years, stent-grafts have been increasingly used in endovascular repair of thoracic and abdominal aneurysm, repair of traumatic subclavian artery and iatrogenic vascular injuries, and in exclusion of peripheral arterial aneurysms[23,24]. However, conventional peripheral vascular stent-grafts are 6-8 mm in diameter and 6 mm in length, making implantation difficult through the tortuous celiac arterial system. Moreover, stent-graft itself is less flexible and seldom used in the celiac system[25,26]. The Jostent, used in our patients for sealing perforation of coronary artery, is a covered stent, ranging 3.5-4.0 mm in diameter and 16-19 mm in length[27]. In the present study, placement of coronary stent-grafts was a useful procedure for the repair of ruptured hepatic artery pseudoaneurysm. However, angiography follow-up was not performed, but hepatic arterial flow was confirmed by Doppler ultrasound examination.

In this study, a high immediate technical success rate was archived using stent-grafts for bleeding hepatic artery pseudoaneurysm, which is consistent with the reported findings[10-15,22]. Bleeding was immediately controlled after placement of stent-grafts and all patients remained stable after the procedure. However, recurrent bleeding occurred in 2 patients, at 16 and 24 h, respectively, after the interventional procedure, possibly due to the dislodgment of stent-grafts.

Of the 7 patients with their bleeding successfully controlled, one died of intra-abdominal sepsis 12 d after the interventional procedure. Consequently, the patency of hepatic artery and stent-grafts without recurrent bleeding was achieved in 6 patients during a mean follow-up time of 10.5 mo (range 4-16 mo).

Placement of stent-grafts with preservation of organ arterial flow, if technically possible, may represent the best treatment option with the following advantages[10-15]. First, it permits immediate and effective control of hemorrhage, thus avoiding emergency surgery, and a second operation can be performed if hemodynamics is stable. Second, placement of stent-grafts is a minimally invasive technique with a low morbidity. Third, placement of stent-grafts preserves end-organ perfusion in the acute stage, thus reducing the risk of organ failure or infarction.

Placement of stent-grafts for hemorrhage from ruptured hepatic artery pseudoaneurysm has several limitations. First, although technical success has been achieved in most published case reports, stent-graft implantation in branches of the celiac trunk is not always possible[10,12,28]. Second, the procedure may lead to rupture of artery because of its eroded and fragile vascular wall, thus requiring emergency surgery. Third, placement of stent-grafts may

lead to in-stent-graft stenosis and occlusion[23,24,27]. It has been recommended that antiplatelet medication should be given after stent-graft deployment in order to prevent in-stent-graft stenosis.

In addition, placement of stent-grafts can control hemorrhage but cannot treat other potential complications[5,16]. To reduce the risk of recurrent hemorrhage, antibiotic therapy for peripancreatic infection and surgical revision of pancreatic or biliary anastomotic leakage may be necessary. Alternatively, pancreatic or biliary leakage can be treated by percutaneous drainage in some patients. In the present study, 6 patients recovered after placement of stent-grafts. Of the 6 patients, 4 underwent CT-guided placement of additional drains, and 2 underwent re-operation due to persistent abscess and PF.

This study has the following limitations: lack of a control group, randomization, and uniformity of evaluation and treatment. In fact, it is almost impossible to perform a prospective randomized study and no statistically significant conclusion could be drawn because of the limited number of patients.

In summary, placement of stent-grafts for acute life-threatening bleeding from hepatic artery pseudoaneurysm is a valuable alternative to embolization and surgical intervention. If technically possible, this technique should be considered the first-line treatment for bleeding from the common and proper hepatic artery, particularly in patients with a non-portal vein. Further data are required to evaluate its technical success rate, complications, and long-term outcome in a larger number of patients.

COMMENTS

Background

Delayed hepatic arterial hemorrhage after pancreaticoduodenectomy (PD) is not a common but a fatal complication, occurring in 7% of all patients. Its ideal management remains unclear and controversial.

Research frontiers

There are many diagnostic and therapeutic options for massive hepatic arterial hemorrhage after PD but no established guidelines are available. Traditional treatment modalities for massive bleeding include re-operation or endovascular catheter techniques such as coil embolization. Surgical exploration and identification of the bleeding vessel can be difficult in acute situations and hazardous because of adhesions and surrounding postsurgical tissue friability. Embolization of the bleeding artery has an immediate impact on patient survival but often excludes the distal circulation, which may have a risk of hepatic ischemia and even fatal hepatic necrosis, particularly in patients with portal vein stenosis or thrombosis. Placement of stent-grafts is a new procedure for control of bleeding without interruption of the distal circulation.

Innovations and breakthroughs

The authors reported the clinical outcome of 9 patients with life-threatening hemorrhage from a ruptured hepatic artery pseudoaneurysm after PD after treatment with a new interventional technique, namely placement of stent-grafts. This technique provides a good alternative option for the control of hemorrhage from ruptured hepatic artery pseudoaneurysm after PD, especially in those who cannot undergo embolization. Although the number of patients was small, the procedure demonstrated a lower mortality than conventional surgical intervention.

Applications

Instead of coil embolization, the authors used stent-graft placement to exclude ruptured hepatic pseudoaneurysm because the patients had an obstructed portal vein prior to massive bleeding. In patients with a non-patent portal vein, embolization of the common or proper hepatic artery may have a high risk of hepatic ischemia and even fatal hepatic necrosis. Placement of stent-grafts in bleeding hepatic artery can immediately and effectively stop the hemorrhage, thus avoiding emergency surgery, and a second operation can be performed if the hemodynamics is stable. Placement of stent-grafts is a minimally invasive technique with a low morbidity. Placement of stent-grafts preserves end-organ perfusion in the acute stage, thus reducing the risk of liver ischemia or failure.

Peer review

The authors describe a small number of patients with life-threatening hemorrhage from ruptured hepatic artery pseudoaneurysm after PD, who were treated with implantation of endovascular stent-grafts. The study is quite interesting and innovative. Placement of stent-grafts is a good procedure for the control of massive bleeding from hepatic artery, especially in those who cannot undergo embolization. Although the number of patients is small, the results can be considered satisfactory and encouraging.

REFERENCES

1 **Limongelli P**, Khorsandi SE, Pai M, Jackson JE, Tait P, Tierris J, Habib NA, Williamson RC, Jiao LR. Management of delayed postoperative hemorrhage after pancreaticoduodenectomy: a meta-analysis. *Arch Surg* 2008; **143**: 1001-1007; discussion 1007

2 **Wente MN**, Veit JA, Bassi C, Dervenis C, Fingerhut A, Gouma DJ, Izbicki JR, Neoptolemos JP, Padbury RT, Sarr MG, Yeo CJ, Büchler MW. Postpancreatectomy hemorrhage (PPH): an International Study Group of Pancreatic Surgery (ISGPS) definition. *Surgery* 2007; **142**: 20-25

3 **Yekebas EF**, Wolfram L, Cataldegirmen G, Habermann CR, Bogoevski D, Koenig AM, Kaifi J, Schurr PG, Bubenheim M, Nolte-Ernsting C, Adam G, Izbicki JR. Postpancreatectomy hemorrhage: diagnosis and treatment: an analysis in 1669 consecutive pancreatic resections. *Ann Surg* 2007; **246**: 269-280

4 **Bassi C**, Dervenis C, Butturini G, Fingerhut A, Yeo C, Izbicki J, Neoptolemos J, Sarr M, Traverso W, Buchler M. Postoperative pancreatic fistula: an international study group (ISGPF) definition. *Surgery* 2005; **138**: 8-13

5 **de Castro SM**, Kuhlmann KF, Busch OR, van Delden OM, Laméris JS, van Gulik TM, Obertop H, Gouma DJ. Delayed massive hemorrhage after pancreatic and biliary surgery: embolization or surgery? *Ann Surg* 2005; **241**: 85-91

6 **Miura F**, Asano T, Amano H, Yoshida M, Toyota N, Wada K, Kato K, Yamazaki E, Kadowaki S, Shibuya M, Maeno S, Furui S, Takeshita K, Kotake Y, Takada T. Management of postoperative arterial hemorrhage after pancreato-biliary surgery according to the site of bleeding: re-laparotomy or interventional radiology. *J Hepatobiliary Pancreat Surg* 2009; **16**: 56-63

7 **Fujii Y**, Shimada H, Endo I, Yoshida K, Matsuo K, Takeda K, Ueda M, Morioka D, Tanaka K, Togo S. Management of massive arterial hemorrhage after pancreatobiliary surgery: does embolotherapy contribute to successful outcome? *J Gastrointest Surg* 2007; **11**: 432-438

8 **Sato N**, Yamaguchi K, Shimizu S, Morisaki T, Yokohata K, Chijiiwa K, Tanaka M. Coil embolization of bleeding visceral pseudoaneurysms following pancreatectomy: the importance of early angiography. *Arch Surg* 1998; **133**: 1099-1102

9 **Baker TA**, Aaron JM, Borge M, Pierce K, Shoup M, Aranha GV. Role of interventional radiology in the management of complications after pancreaticoduodenectomy. *Am J Surg* 2008; **195**: 386-390; discussion 390

10 **Won YD**, Ku YM, Kim KT, Kim KH, Kim JI. Successful management of a ruptured hepatic artery pseudoaneurysm with a stent-graft. *Emerg Radiol* 2009; **16**: 247-249

11 **Sasaki K**, Ueda K, Nishiyama A, Yoshida K, Sako A, Sato M, Okumura M. Successful utilization of coronary covered stents to treat a common hepatic artery pseudoaneurysm secondary to pancreatic fistula after Whipple's procedure: report of a case. *Surg Today* 2009; **39**: 68-71

12 **Rossi M**, Rebonato A, Greco L, Citone M, David V. Endovascular exclusion of visceral artery aneurysms with stent-grafts:

technique and long-term follow-up. *Cardiovasc Intervent Radiol* 2008; **31**: 36-42
13 **Pasklinsky G**, Gasparis AP, Labropoulos N, Pagan J, Tassiopoulos AK, Ferretti J, Ricotta JJ. Endovascular covered stenting for visceral artery pseudoaneurysm rupture: report of 2 cases and a summary of the disease process and treatment options. *Vasc Endovascular Surg* 2008; **42**: 601-606
14 **Ichihara T**, Sato T, Miyazawa H, Shibata S, Hashimoto M, Ishiyama K, Yamamoto Y. Stent placement is effective on both postoperative hepatic arterial pseudoaneurysm and subsequent portal vein stricture: a case report. *World J Gastroenterol* 2007; **13**: 970-972
15 **Stoupis C**, Ludwig K, Inderbitzin D, Do DD, Triller J. Stent grafting of acute hepatic artery bleeding following pancreatic head resection. *Eur Radiol* 2007; **17**: 401-408
16 **Gebauer T**, Schulz HU, Tautenhahn J, Halloul Z, Effenberger O, Lippert H, Bürger T. [Interventional and vascular surgical management for inflammatory arrosion hemorrhage from visceral arteries following pancreatic surgery] *Chirurg* 2004; **75**: 1021-1028
17 **Tessier DJ**, Fowl RJ, Stone WM, McKusick MA, Abbas MA, Sarr MG, Nagorney DM, Cherry KJ, Gloviczki P. Iatrogenic hepatic artery pseudoaneurysms: an uncommon complication after hepatic, biliary, and pancreatic procedures. *Ann Vasc Surg* 2003; **17**: 663-669
18 **Bassi C**, Falconi M, Salvia R, Mascetta G, Molinari E, Pederzoli P. Management of complications after pancreaticoduodenectomy in a high volume centre: results on 150 consecutive patients. *Dig Surg* 2001; **18**: 453-457; discussion 458
19 **Tsirlis T**, Vasiliades G, Koliopanos A, Kopanakis N, Katseli A, Tsipras H, Margaris H. Pancreatic leak related hemorrhage following pancreaticoduodenectomy. A case series. *JOP* 2009; **10**: 492-495
20 **Khorsandi SE**, Limongelli P, Jackson JE, Tait P, Williamson RC, Habib NA, Jiao LR. Management of delayed arterial hemorrhage after pancreaticoduodenectomy. A case series. *JOP* 2008; **9**: 172-178
21 **Teramoto K**, Kawamura T, Takamatsu S, Noguchi N, Arii S. A case of hepatic artery embolization and partial arterialization of the portal vein for intraperitoneal, hemorrhage after a pancreaticoduodenectomy. *Hepatogastroenterology* 2003; **50**: 1217-1219
22 **Makowiec F**, Riediger H, Euringer W, Uhl M, Hopt UT, Adam U. Management of delayed visceral arterial bleeding after pancreatic head resection. *J Gastrointest Surg* 2005; **9**: 1293-1299
23 **Xenos ES**, Freeman M, Stevens S, Cassada D, Pacanowski J, Goldman M. Covered stents for injuries of subclavian and axillary arteries. *J Vasc Surg* 2003; **38**: 451-454
24 **Baltacioğlu F**, Cimşit NC, Cil B, Cekirge S, Ispir S. Endovascular stent-graft applications in latrogenic vascular injuries. *Cardiovasc Intervent Radiol* 2003; **26**: 434-439
25 **Tielliu IF**, Verhoeven EL, Zeebregts CJ, Prins TR, Oranen BI, van den Dungen JJ. Endovascular treatment of iliac artery aneurysms with a tubular stent-graft: mid-term results. *J Vasc Surg* 2006; **43**: 440-445
26 **White R**, Krajcer Z, Johnson M, Williams D, Bacharach M, O'Malley E. Results of a multicenter trial for the treatment of traumatic vascular injury with a covered stent. *J Trauma* 2006; **60**: 1189-1195; discussion 1195-1196
27 **Lansky AJ**, Yang YM, Khan Y, Costa RA, Pietras C, Tsuchiya Y, Cristea E, Collins M, Mehran R, Dangas GD, Moses JW, Leon MB, Stone GW. Treatment of coronary artery perforations complicating percutaneous coronary intervention with a polytetrafluoroethylene-covered stent graft. *Am J Cardiol* 2006; **98**: 370-374
28 **Larson RA**, Solomon J, Carpenter JP. Stent graft repair of visceral artery aneurysms. *J Vasc Surg* 2002; **36**: 1260-1263

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CASE REPORT

Right anterior segmental hepatic duct emptying directly into the cystic duct in a living donor

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Abstract

A 35-year-old mother was scheduled to be the living donor for liver transplantation to her second son, who suffered from biliary atresia complicated with biliary cirrhosis at the age of 2 years. The operative plan was to recover the left lateral segment of the mother's liver for living donor transplantation. With the use of cholangiography at the time of surgery, we found the right anterior segmental duct (RASD) emptying directly into the cystic duct, and the catheter passed into the RASD. After repairing the incision in the cystic duct, transplantation was successfully performed. Her postoperative course was uneventful. Biliary anatomical variations were frequently encountered, however, this variation has very rarely been reported. If the RASD was divided, the repair would be very difficult because the duct will not dilate sufficiently in an otherwise healthy donor. Meticulous preoperative evaluation of the living donor's biliary anatomy, especially using magnetic resonance cholangiography and careful intraoperative techniques, is important to prevent bile duct injury and avoid the risk to the healthy donor.

Key words: Bile duct injury; Cystohepatic duct; Intraoperative cholangiography; Living liver transplantation; Bile duct injury; Magnetic resonance cholangiography

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Ishiguro Y, Hyodo M, Fujiwara T, Sakuma Y, Hojo N, Mizuta K, Kawarasaki H, Lefor AT, Yasuda Y. Right anterior segmental hepatic duct emptying directly into the cystic duct in a living donor. *World J Gastroenterol* 2010; 16(29): 3723-3726 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i29/3723.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i29.3723

INTRODUCTION

Living donor liver transplantation (LDLT) is accepted around the world with the shortage of suitable deceased donors[1-4]. The safety of the living donor, an otherwise healthy individual, is important in the ethical considerations of performing LDLT[5]. Morbidity in living donors is not rare[3,6,7]. Preoperative imaging studies are needed not only to evaluate the donor's condition but also to exclude unsuitable candidates. The liver volume, condition of the liver parenchyma, and vascular anatomy are relatively easy to assess through imaging studies especially using multi-detector row computed tomography (MDCT). Variations in biliary anatomy are common,

and in order to avoid intraoperative biliary injury, precise preoperative imaging studies are needed. However, it is difficult to evaluate biliary anatomy because of the complicated nature of various imaging modalities. There is no consensus for preoperative imaging to evaluate the donor's biliary anatomy. Even today intraoperative cholangiography (IOC) has a very important role in the assessment of a donor's biliary anatomy.

We identified a rare biliary anatomic variant during LDLT. The donor had the right anterior segmental duct (RASD) emptying directly into the cystic duct. In this report, we present this rare but important anatomic variant and discuss preoperative biliary imaging and the procedure for IOC.

CASE REPORT

A 35-year-old mother was scheduled to be the living donor for liver transplantation to her second son, who suffered from biliary atresia complicated with biliary cirrhosis at the age of 2 years. There were no remarkable findings in her family or past medical history. On physical examination, she had a healed operative scar in the lower abdomen as a result of two caesarean section procedures. A preoperative medical evaluation was performed and her liver volume, condition of the liver parenchyma, and hepatic vascular anatomy made her a suitable living donor. Congenital absence of the right kidney and slight prolongation of the activated partial thromboplastin time were found, which were not considered as contraindications for donor surgery. At that time, preoperative imaging of the biliary system was not performed routinely in our institution. LDLT was then performed, using an upper midline incision to recover the left lateral segment. After intraoperative ultrasound examination, cholecystectomy was performed. Before the gallbladder was removed, IOC was attempted through the incision in the cystic duct based on the conventional practice. However, the catheter would not pass easily into the cystic duct. Instead, it passed through a small hole in the side wall of the cystic duct. Using cholangiography, we found that the RASD emptied directly into the cystic duct with the catheter inserted into the RASD (Figure 1A), and the right posterior segmental duct joined the left hepatic duct. The incision in the cystic duct was repaired carefully to prevent stenosis. After removing the left lateral segment, another intraoperative cholangiogram was performed through the stump of the left hepatic duct and no stenosis at the confluence of the RASD and cystic duct was seen (Figure 1B). Her postoperative course was uneventful.

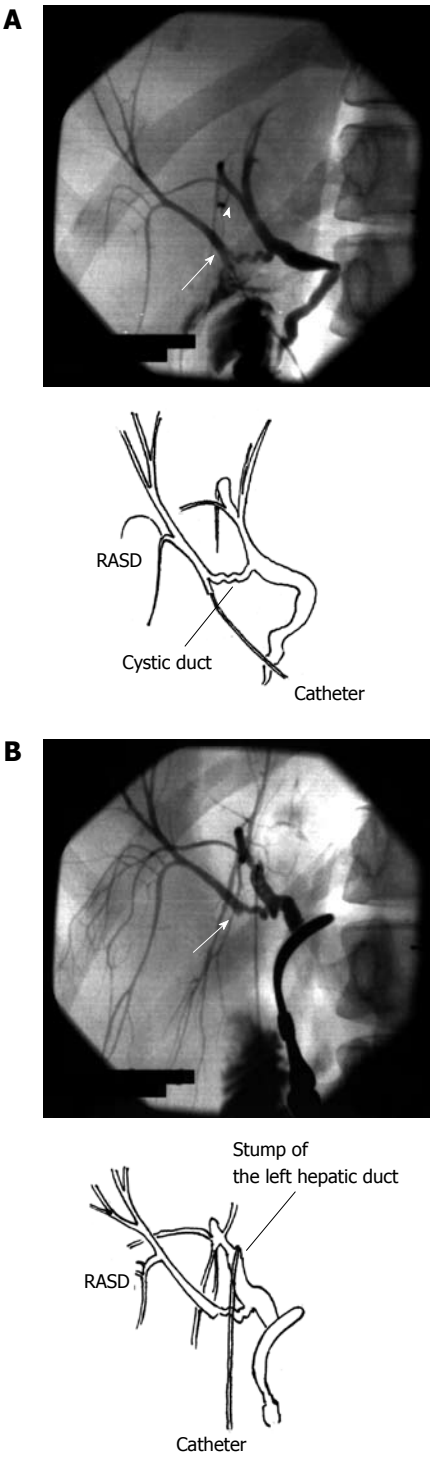

Figure 1 Intraoperative cholangiography. A: The right anterior segmental duct (RASD) emptying into the cystic duct is seen (arrow). The catheter is in the RASD. The right posterior segmental duct joining the left hepatic duct is also shown (arrowhead); B: After the left lateral segment was recovered, no stenosis was seen at the site of the repaired cystic duct (arrow). Cholangiography was performed through the stump of the left hepatic duct.

DISCUSSION

Biliary anatomical variants are frequently encountered[8], however, the variation reported in this case is very rare[9-12]. Champetier *et al*[13] stated that "The cystohepatic ducts drain the entirety of a hepatic territory of variable extent into the cystic duct or gallbladder", and they also emphasized the danger of these variants when performing a cholecystectomy.

The possibility of very rare biliary variations and the risks for the donor must be discussed in detail when ob-

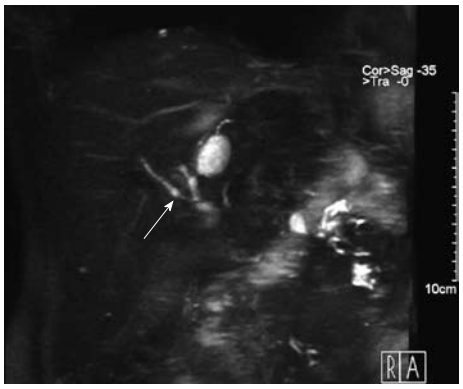

Figure 2 Magnetic resonance cholangiography was performed 3 mo postoperatively. The right anterior segmental duct and cystic duct are clearly seen (arrow).

taining informed consent. The living donor will be at risk, but continued efforts must be made to reduce the donor's morbidity as much as possible. In this case, we encountered a rare variant of biliary duct anatomy, but avoided injury to the RASD.

One of the ethical obligations of LDLT is to carefully protect the safety of the donor[5]. If the RASD was divided, the repair would be very difficult because the duct may not dilate sufficiently in an otherwise healthy donor. The donor might then suffer severe morbidities. To reduce the donor's risk, meticulous preoperative evaluation of the living donor's biliary anatomy is important. The transplant surgeons must be aware of biliary anatomic variants to prevent any intraoperative bile duct injury and minimize the risk to the donor. However, there is no single examination to demonstrate all biliary conditions expeditiously. Endoscopic retrograde cholangiography has some risks, such as the development of biliary injury, pancreatitis, and other conditions. MDCT is a breakthrough not only for demonstrating vascular anatomy but also to clearly depict the anatomy of the biliary system using biliary contrast material[14,15]. This modality also carries the risk of an allergic reaction. The possibility of a severe allergic reaction using vascular contrast material is only 0.04%[16]. On the other hand, biliary contrast materials have more risks of severe allergic reactions[17] and occur in up to 0.2% of patients according to the information from Japanese manufacturers. Dual enhanced MDCT is thought to be a very useful and accurate modality[14,15], but contains the relatively higher risk of an allergic reaction. Magnetic resonance cholangiography (MRC) is used in the preoperative examination of donors in some institutions[18,19]. However, MRC has relatively lower spatial resolution and a thin cystohepatic duct might not be accurately depicted. Postoperatively, the donor reported in this study was examined by MRC and the RASD was observed (Figure 2). The performance of this procedure was influenced by the intraoperative findings in this patient, but also led us to use MRC routinely in the preoperative evaluation of the donor's biliary anatomy to maximize the safety of the donors.

Careful intraoperative technique is also needed to improve the donor patient's safety, by assuring appropriate management of aberrant biliary branches. IOC is an essential modality to complete the donor transplantation procedure. In many cases, IOC is performed through an incision in the cystic duct; however, it may be hazardous to incise the cystic duct in a case as reported here. Therefore, we recommend using a modified IOC, incising the area of Hartmann's pouch. We believe that this method can reduce the possibility of injuring a very small but important biliary duct that might be not seen on preoperative imaging studies. It has been reported that IOC has some risks of allergic reactions[20], we believe that IOC itself is safe because little contrast material enters the systemic circulation. Using the knowledge gained from preoperative imaging and careful IOC, the safety of the donor is maximized.

In conclusion, to prevent injury of the bile ducts during the donor surgery of LDLT, preoperative delineation of the biliary anatomy is essential. Considering the donor's likelihood of suffering an allergic reaction, and the spatial resolution of various imaging modalities, we recommend MRC as the ideal preoperative biliary imaging study for the donors. IOC also plays an important role in the management of these donors. To perform IOC more carefully, we recommend that Hartmann's pouch should be incised first. By doing this we can help avoid transection of fine biliary branches that are not revealed during preoperative imaging, thus minimizing the likelihood of this avoidable complication.

REFERENCES

1 **Broelsch CE**, Burdelski M, Rogiers X, Gundlach M, Knoefel WT, Langwieler T, Fischer L, Latta A, Hellwege H, Schulte FJ. Living donor for liver transplantation. *Hepatology* 1994; **20**: 49S-55S
2 **Rogiers X**, Burdelski M, Broelsch CE. Liver transplantation from living donors. *Br J Surg* 1994; **81**: 1251-1253
3 **Broelsch CE**, Malagó M, Testa G, Valentin Gamazo C. Living donor liver transplantation in adults: outcome in Europe. *Liver Transpl* 2000; **6**: S64-S65
4 **Raia S**, Nery JR, Mies S. Liver transplantation from live donors. *Lancet* 1989; **2**: 497
5 **Malagó M**, Burdelski M, Broelsch CE. Present and future challenges in living related liver transplantation. *Transplant Proc* 1999; **31**: 1777-1781
6 **Nery JR**, Fragulidis GP, Scagnelli T, Weppler D, Webb MG, Khan MF, Tzakis AG. Donor biliary variations: an overlooked problem? *Clin Transplant* 1997; **11**: 582-587
7 **Grewal HP**, Thistlewaite JR Jr, Loss GE, Fisher JS, Cronin DC, Siegel CT, Newell KA, Bruce DS, Woodle ES, Brady L, Kelly S, Boone P, Oswald K, Millis JM. Complications in 100 living-liver donors. *Ann Surg* 1998; **228**: 214-219
8 **Puente SG**, Bannura GC. Radiological anatomy of the biliary tract: variations and congenital abnormalities. *World J Surg* 1983; **7**: 271-276
9 **Heloury Y**, Leborgne J, Rogez JM, Robert R, Lehur PA, Pannier M, Barbin JY. Radiological anatomy of the bile ducts based on intraoperative investigation in 250 cases. *Anat Clin* 1985; **7**: 93-102
10 **Sussman SK**, Hall FM, Elboim CM. Radiographic assessment of anomalous bile ducts. *Gastrointest Radiol* 1986; **11**: 269-272

11 **Reid SH**, Cho SR, Shaw CI, Turner MA. Anomalous hepatic duct inserting into the cystic duct. *AJR Am J Roentgenol* 1986; **147**: 1181-1182
12 **Benson MD**, Strong RW. Aberrant hepatic ducts. *Australas Radiol* 1988; **32**: 348-355
13 **Champetier J**, Létoublon C, Alnaasan I, Charvin B. The cystohepatic ducts: surgical implications. *Surg Radiol Anat* 1991; **13**: 203-211
14 **Schroeder T**, Malagó M, Debatin JF, Testa G, Nadalin S, Broelsch CE, Ruehm SG. Multidetector computed tomographic cholangiography in the evaluation of potential living liver donors. *Transplantation* 2002; **73**: 1972-1973
15 **Schroeder T**, Nadalin S, Stattaus J, Debatin JF, Malagó M, Ruehm SG. Potential living liver donors: evaluation with an all-in-one protocol with multi-detector row CT. *Radiology* 2002; **224**: 586-591
16 **Katayama H**, Yamaguchi K, Kozuka T, Takashima T, Seez P, Matsuura K. Adverse reactions to ionic and nonionic contrast media. A report from the Japanese Committee on the Safety of Contrast Media. *Radiology* 1990; **175**: 621-628
17 **Cheng YF**, Lee TY, Chen CL, Huang TL, Chen YS, Lui CC. Three-dimensional helical computed tomographic cholangiography: application to living related hepatic transplantation. *Clin Transplant* 1997; **11**: 209-213
18 **Kapoor V**, Peterson MS, Baron RL, Patel S, Eghtesad B, Fung JJ. Intrahepatic biliary anatomy of living adult liver donors: correlation of mangafodipir trisodium-enhanced MR cholangiography and intraoperative cholangiography. *AJR Am J Roentgenol* 2002; **179**: 1281-1286
19 **Ayuso JR**, Ayuso C, Bombuy E, De Juan C, Llovet JM, De Caralt TM, Sánchez M, Pagés M, Bruix J, García-Valdecasas JC. Preoperative evaluation of biliary anatomy in adult live liver donors with volumetric mangafodipir trisodium enhanced magnetic resonance cholangiography. *Liver Transpl* 2004; **10**: 1391-1397
20 **Mortelé KJ**, Cantisani V, Troisi R, de Hemptinne B, Silverman SG. Preoperative liver donor evaluation: Imaging and pitfalls. *Liver Transpl* 2003; **9**: S6-S14

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CASE REPORT

Ultrasonography of sclerosing angiomatoid nodular transformation in the spleen

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Abstract

We report three rare cases of sclerosing angiomatoid nodular transformation (SANT) in the spleen. We compared the conventional and contrast-enhanced ultrasonographic appearance. The conventional sonographic examinations exhibited solitary lesions without common respects, while contrast-enhanced ultrasonography (CEUS) revealed nodular appearance mimicking its pathologic characteristics. It suggests that CEUS can provide morphologic information for diagnosing SANT.

Key words: Sclerosing angiomatoid nodular transformation; Contrast-enhanced ultrasonography; Ultrasound

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INTRODUCTION

Sclerosing angiomatoid nodular transformation (SANT) is a rare benign lesion. Martel named it for its pathological characteristics[1]. Its particular morphologic appearance, immunophenotype, and benign clinical course indicate that it is a distinctive non-neoplastic vascular lesion in the spleen. We report the ultrasonographic images in three cases of SANT confirmed by splenectomy. Two of them also underwent contrast-enhanced ultrasonography (CEUS). We used a second-generation contrast agent, SonoVue (Bracco, Italy) at a dose of 1.2 mL[2].

CASE REPORT

Case 1

A 36-year-old man was found to have a splenic mass by a routine medical examination without any symptoms. Physical examination found nothing remarkable. No cervical or inguinal lymphadenopathy was found. The laboratory test results were as follows: hemoglobin 130 g/L, Ery 4.1 T/L, WBC 14.5 g/L, neutrophils 12.0 g/L and platelets 174 g/L, and all other data were within normal limits. Abdominal ultrasonography (US) was done using a 3.5C ultrasound transducer attached to a LOGIQ 5 Expert; it demonstrated a 4.2 cm × 3.7 cm well-demarcated, hypoechoic lesion in the spleen (Figure 1). The patient also underwent an MRI examination. On T1- and T2-weighted images, heterogeneous hypo-signal intensity with peripheral and septa enhancement, especially at the delayed phase, was displayed. The patient underwent splenectomy. In the surgery, the mass was found to have an integrated envelope and a heterogeneous cut surface. A few days after the surgery, his WBC and neutrophils became normal.

Case 2

A 37-year-old woman was referred to abdominal ultrasound examination because of pain in the left upper quadrant. Lesions were found both in her liver and spleen. Pro-

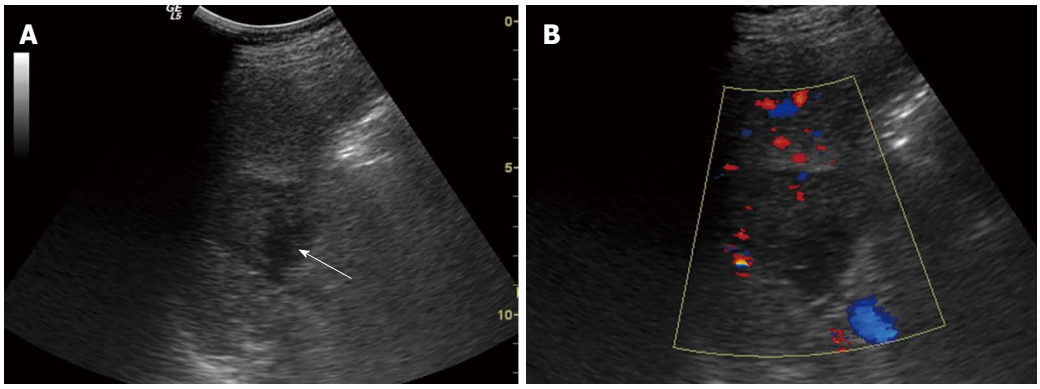

Figure 1 Conventional sonographic findings of sclerosing angiomatoid nodular transformation in a 36-year-old man. A: B mode ultrasonography demonstrates a hypoechoic lesion (arrow) in the spleen; B: Color Doppler flow imaging shows a low color-flow signal in the lesion.

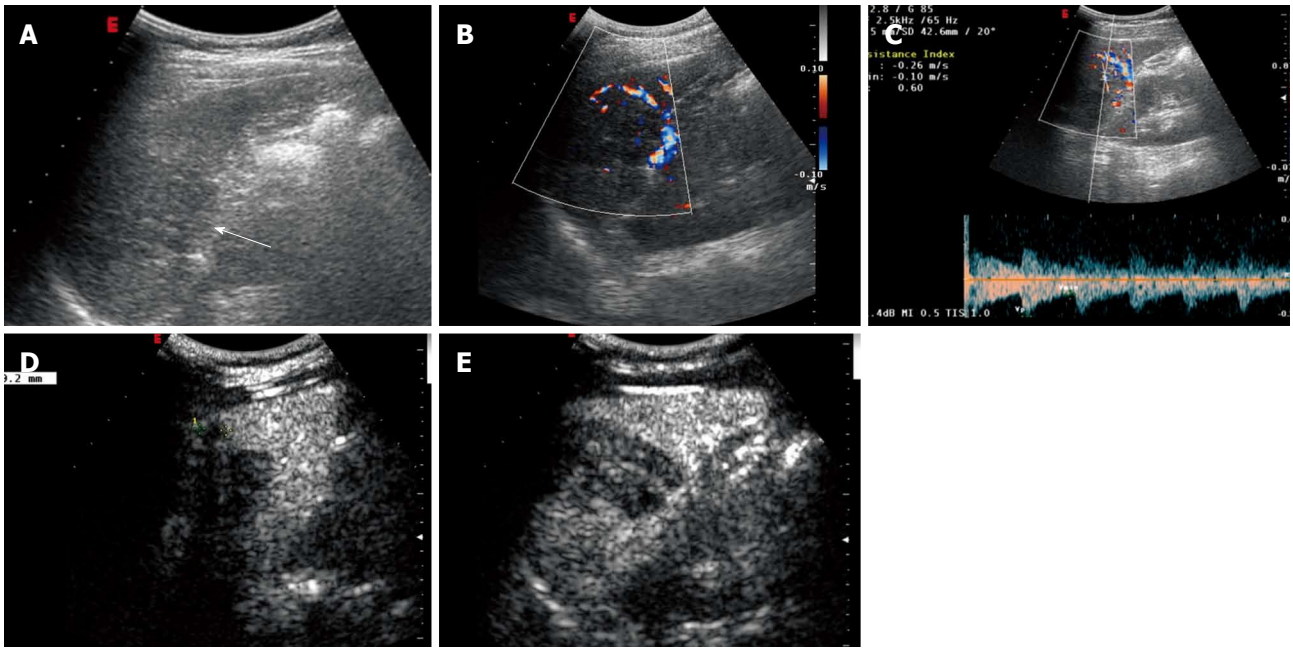

Figure 2 Conventional ultrasonography and contrast-enhanced ultrasonography findings of sclerosing angiomatoid nodular transformation in a 37-year-old woman with pain in the left upper quadrant. A-C: A heterogeneous hypoechoic lesion (arrow) in the spleen, with blood-flow signals in the peripheral area; D: There was a 0.9 cm homologous nodule attaching to the side of the lesion that became evident after being enhanced (2 min 48 s after injection); E: The lesion was enhanced homogeneously.

liferative disease of metastasis was taken into consideration during the differentiation process. Physical examination found nothing remarkable. No cervical or inguinal lymphadenopathy was observed. The laboratory tests yielded the following results: hemoglobin 120 g/L, Ery 3.88 T/L, WBC 4.6 g/L, neutrophils 3.2 g/L, platelets 214 g/L, CEA 1.16 ng/mL, AFP 2.4 ng/mL and CA19-9 6.79 U/mL. Other data were all within normal limits. Abdominal ultrasonography was done using a CA430E5-2 ultrasound transducer attached to a Technos ESAOTE DU 8; it demonstrated a 5.2 cm × 3.6 cm heterogeneous hypoechoic lesion in the spleen with an unclear margin. Color Doppler flow imaging (CDFI) showed blood-flow signals in the peripheral area of the lesion and resistance index (RI) was 0.66 (Figure 2A-C). The lesion was enhanced in the diffuse pattern from 11 s after injection, and appeared to be homogeneously hyperechoic in comparison with the splenic parenchyma in 21 s. It turned out to be isoechoic in 4 s and hypoechoic in 30 s after the injection (Figure 2E). The lesion showed a persistent enhancement up to about 7 min. Another 0.9 cm × 0.9 cm homologous nodule was found attaching to the lesion which became evident after being enhanced (Figure 2D). In order to reach a final diagnosis, the patient underwent surgical excision of the spleen and part of the liver. In the surgery, a splenic mass was firm with a clear margin. The pathological result is a SANT of the spleen and hemangiomas in the liver.

Case 3

A 39-year-old man was admitted to the hospital because of a left upper quadrant mass of unknown cause. Splenomegaly was suspected by physical examination. No cervi-

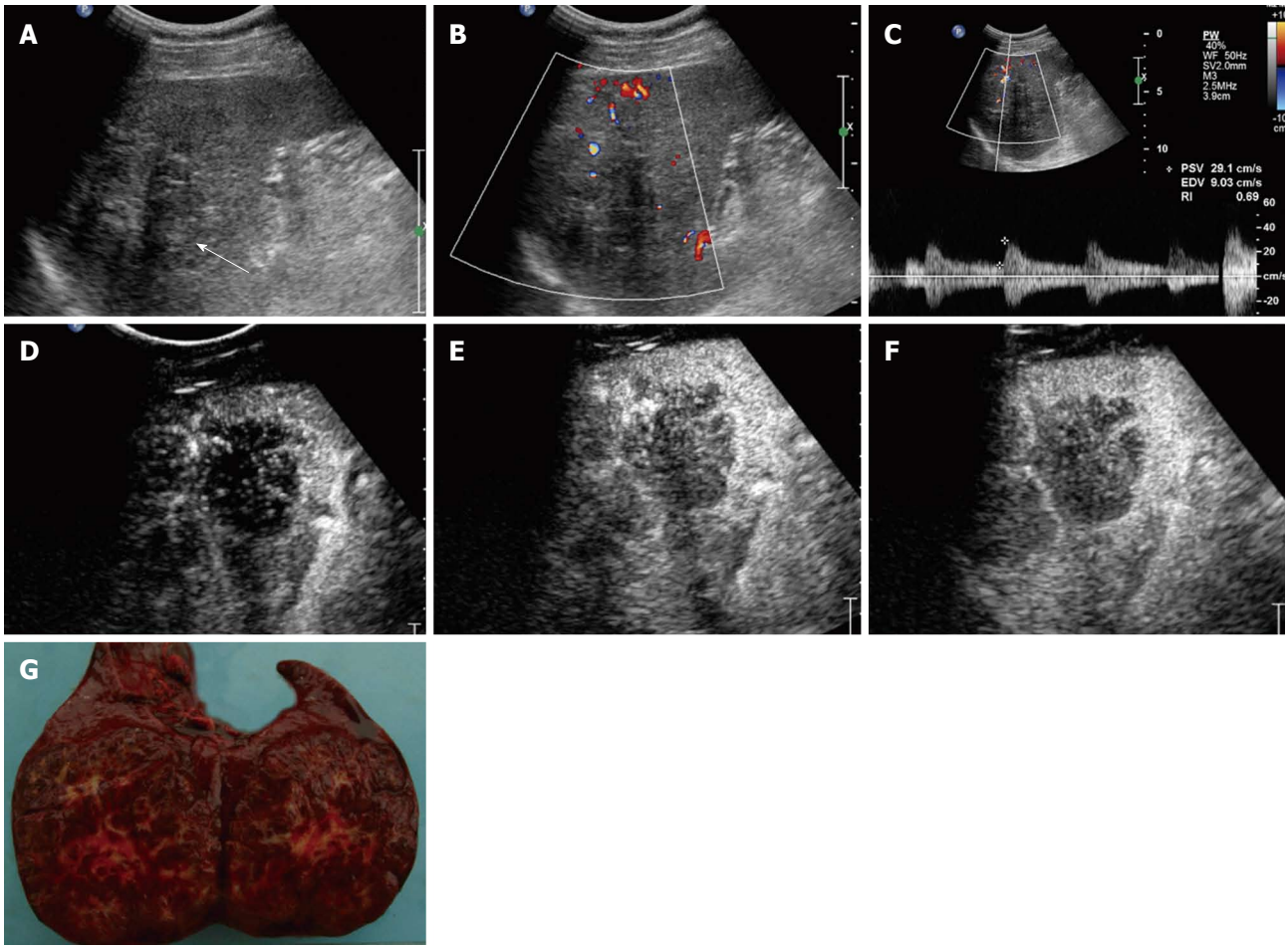

Figure 3 Conventional ultrasonography and contrast-enhanced ultrasonography findings of sclerosing angiomatoid nodular transformation in a 39-year-old man. A: A hypoechoic lesion (arrow) in the spleen; B and C: Color Doppler flow imaging shows branch-shaped and wire-like blood-flow signals inside and spot-shaped blood-flow signals in the periphery of the lesion; D: The lesion was enhanced in the branch-shaped diffuse pattern (15 s after injection); E: It presented heterogeneous lobular appearance when reaching its peak enhancement; F: Lots of septa inside the lesion; G: Surface of the lesion found during the operation.

cal or inguinal lymphadenopathy was found. The laboratory test results were: hemoglobin 123 g/L, Ery 4.23 T/L, WBC 6.7 g/L, neutrophils 4.6 g/L, platelets 167 g/L, CEA 0.56 ng/mL, AFP 2.4 ng/mL and CA19-9 3.6 U/mL. All other laboratory tests were within normal limits. Abdominal ultrasonography was done using a C5-2 ultrasound transducer attached to a Philips IU22; it demonstrated a 7.0 cm × 6.2 cm well-demarcated, hypoechoic lesion in the spleen. CDFI showed branch-shaped and wire-like blood-flow signals inside and spot-shaped blood-flow signals in the periphery of the lesion. RI was 0.50-0.66 (Figure 3A-C). The lesion was enhanced in the branch-shaped diffuse pattern from 12 s after injection (Figure 3D). It presented heterogeneous lobular appearance when reaching its peak enhancement in 23 s (Figure 3E). We also detected lots of septa inside the lesion (Figure 3F). It became hypoechoic in 37 s in comparison with the splenic parenchyma. The lesion showed a persistent enhancement up to about 3 min. Its enhancement intensity was less than that of the spleen during the whole enhancement process. Its structure and margin were clearer than those of conventional ultrasonographic images. The patient also received CT examination, which revealed a low-density mass with mildly septa enhancement. The final diagnosis of SANT was confirmed by operation (Figure 3G).

DISCUSSION

SANT is a rare mild vascular lesion in the spleen. The age of our patients ranged from 36 to 39 years. Two of them were asymptomatic (Case 1 and Case 3). The lesions were usually found incidentally. Few patients presented with abdominal discomfort or splenomegaly. Its etiology is still unclear. Concurrent diseases included chronic lymphocytic leukemia, lung squamous cell carcinoma, colonic carcinoma and some other diseases[1]. We cannot confirm whether hepatic hemangioma as shown in Case 2 is related to SANT. The lesion presented as a solitary mass measuring 3-17 cm in greatest diameter as reported before[1]. And usually it has a clear margin with a normal splenic parenchyma. The gross section shows splenic angiomatoid nodules as altered red pulp tissue is entrapped by a non-neoplastic stromal proliferative process[3]. SANT is composed of small blood vessels of three typically distinct immunophenotypes: cord capillaries (CD34+/CD8-/CD31+), sinusoids (CD34-/CD8+/

CD31+), and small veins (CD34-/CD8-/CD31+). It can be differentiated from other vascular tumors or tumor-like lesions, including hemangioma, littoral cell angioma (LCA), inflammatory pseudotumor (IPT) and follicular dendritic cell tumor (FDC), by immunohistochemical examinations.

Sonographic findings of our cases are described as follows: All the lesions were solitary and heterogeneous. Their greatest diameters were 3-7 cm. Two of them were hypoechoic and the other lesion was hyperechoic. Linear and slit-like hyperechoic septa was presented in one lesion (Case 3). The ratio of having a clear and unclear margin is 2:1. The color-Doppler showed arterial flow signals in all the lesions and their RIs were low. The contrast-enhanced images in the two lesions had something in common. The contrast media entered the lesions 1-2 s later than the splenic parenchyma. And they came out of the lesions earlier than that of the spleen. The lesions were both enhanced in diffuse pattern. One lesion (Case 3) presented a heterogeneous lobule with lots of septa inside at the peak enhancement. An extra 0.9 cm × 0.9 cm nodule attaching to the previously-found lesion was detected in Case 2 during the enhancement process.

Usually, we consider hyperechoic lesions in the spleen as benign ones on US images such as hemangioma. Malignant lesions are mostly hypoechoic and occur most frequently in lymphomas (80% of focal lesions)[4]. On conventional US images, the three lesions presented with different echogenicity. They were lack of consistency in their boundaries and shapes. An assessment of conventional US images does not provide any differential diagnostic clues. Additionally, color-Doppler technique is only capable of detecting some obvious color flow signals in big solid masses in the spleen. The images of conventional US examination are not characteristic.

We found few cases reporting the behavior of SANT with ultrasonography in the literature. Gutzeit reported a case of SANT[5], which had a hypoechoic halo, predominantly vascular on conventional US images and a "spoke wheel" pattern on CEUS images. In our cases, the heterogeneous lobule and lots of septa inside at the peak enhancement in Case 3 are similar to the "spoke wheel" pattern, but no vascular halo was present. The angiomatoid nodules are frequently delimited by concentric bands of collagen fibers, and there is always an inflamed fibrocellular to sclerotic stroma between the nodules at low magnification. Some exhibited fibrin deposition in the peripheral zone of the nodules. CEUS has shown to be useful in observing nodules whose contrast to the spleen parenchyma was enhanced after injection of SonoVue. Because of its multinodular angiomatoid appearance, SANT was described by Rosai[6] as the term "multinodular hemangioma". In conclusion, CEUS is a new imaging technique that provides direct visualization of vessel structure and morphologic characteristics of the lesions.

Pathologically, SANT has been thought to be a variant of IPT. SANT has fewer inflammatory cells and myofibroblasts than those in IPT. Martel *et al*[1] reported one case of fever and three cases of high erythrocyte sedimentation among 25 cases of SANT. One patient (Case 1) also had high WBC and neutro phils before the operation. Consequently, the US appearances of the lesions reported above are between those of IPT and hemangioma. It can explain the diversity of its echogenicity, morphology and boundary.

REFERENCES

1 **Martel M**, Cheuk W, Lombardi L, Lifschitz-Mercer B, Chan JK, Rosai J. Sclerosing angiomatoid nodular transformation (SANT): report of 25 cases of a distinctive benign splenic lesion. *Am J Surg Pathol* 2004; **28**: 1268-1279

2 **Lim AK**, Patel N, Eckersley RJ, Taylor-Robinson SD, Cosgrove DO, Blomley MJ. Evidence for spleen-specific uptake of a microbubble contrast agent: a quantitative study in healthy volunteers. *Radiology* 2004; **231**: 785-788

3 **Lee JC**, Lien HC, Hsiao CH. Coexisting sclerosing angiomatoid nodular transformation of the spleen with multiple calcifying fibrous pseudotumors in a patient. *J Formos Med Assoc* 2007; **106**: 234-239

4 **Woszczyk D**, Kołodziej-Jaskuła A, Mykała-Cieśla J, Bal A, Łabuzek K, Gasińska T. Focal lesions in the ultrasonographic examination of the spleen as a symptom of various disease statuses: literature survey and case descriptions. *Med Sci Monit* 2006; **12**: CS67-CS73

5 **Gutzeit A**, Stuckmann G, Dommann-Scherrer C. Sclerosing angiomatoid nodular transformation (SANT) of the spleen: sonographic finding. *J Clin Ultrasound* 2009; **37**: 308-311

6 **Rosai J**. Spleen. In: Rosai and Ackerman's Surgical Pathology. 9th ed. Edinburgh: Mosby, 2004: 20-35

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Meetings

Events Calendar 2010

January 25-26
Tamilnadu, India
International Conference on Medical Negligence and Litigation in Medical Practice

January 25-29
Waikoloa, HI, United States
Selected Topics in Internal Medicine

January 26-27
Dubai, United Arab Emirates
2nd Middle East Gastroenterology Conference

January 28-30
Hong Kong, China
The 1st International Congress on Abdominal Obesity

February 11-13
Fort Lauderdale, FL, United States
21th Annual International Colorectal Disease Symposium

February 26-28
Carolina, United States
First Symposium of GI Oncology at The Caribbean

March 04-06
Bethesda, MD, United States
8th International Symposium on Targeted Anticancer Therapies

March 05-07
Peshawar, Pakistan
26th Pakistan Society of Gastroenterology & Endoscopy Meeting

March 09-12
Brussels, Belgium
30th International Symposium on Intensive Care and Emergency Medicine

March 12-14
Bhubaneswar, India
18th Annual Meeting of Indian National Association for Study of the Liver

March 23-26
Cairo, Egypt
14th Pan Arab Conference on Diabetes PACD14

March 25-28
Beijing, China
The 20th Conference of the Asian Pacific Association for the Study of the Liver

March 27-28
San Diego, California, United States
25th Annual New Treatments in Chronic Liver Disease

April 07-09
Dubai, United Arab Emirates
The 6th Emirates Gastroenterology and Hepatology Conference, EGHC 2010

April 14-17
Landover, Maryland, United States
12th World Congress of Endoscopic Surgery

April 14-18
Vienna, Austria
The International Liver Congress™ 2010

April 28-May 01
Dubrovnik, Croatia
3rd Central European Congress of surgery and the 5th Croatian Congress of Surgery

May 01-05
New Orleans, LA, United States
Digestive Disease Week Annual Meeting

May 06-08
Munich, Germany
The Power of Programming: International Conference on Developmental Origins of Health and Disease

May 15-19
Minneapolis, MN, United States
American Society of Colon and Rectal Surgeons Annual Meeting

June 04-06
Chicago, IL, United States
American Society of Clinical Oncologists Annual Meeting

June 09-12
Singapore, Singapore
13th International Conference on Emergency Medicine

June 14
Kosice, Slovakia
Gastro-intestinal Models in the Research of Probiotics and Prebiotics-Scientific Symposium

June 16-19
Hong Kong, China
ILTS: International Liver Transplantation Society ILTS Annual International Congress

June 20-23
Mannheim, Germany
16th World Congress for Bronchoesophagology-WCBE

June 25-29
Orlando, FL, United States
70th ADA Diabetes Scientific Sessions

August 28-31
Boston, Massachusetts, United States
10th OESO World Congress on Diseases of the Oesophagus 2010

September 10-12
Montreal, Canada
International Liver Association's Fourth Annual Conference

September 11-12
La Jolla, CA, United States
New Advances in Inflammatory Bowel Disease

September 12-15
Boston, MA, United States
ICAAC: Interscience Conference on Antimicrobial Agents and Chemotherapy Annual Meeting

September 16-18
Prague, Czech Republic
Prague Hepatology Meeting 2010

September 23-26
Prague, Czech Republic
The 1st World Congress on Controversies in Gastroenterology & Liver Diseases

October 07-09
Belgrade, Serbia
The 7th Biannual International Symposium of Society of Coloproctology

October 15-20
San Antonio, TX, United States
ACG 2010: American College of Gastroenterology Annual Scienitfic Meeting

October 23-27
Barcelona, Spain
18th United European Gastroenterology Week

October 29-November 02
Boston, Massachusetts, United States
The Liver Meeting® 2010--AASLD's 61st Annual Meeting

November 13-14
San Francisco, CA, United States
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Acknowledgments

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2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

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

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

No volume or issue

9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

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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