World Journal of Gastroenterology®

Volume 12 Number 48
December 28, 2006

Supported by NSFC
2005-2006

National Journal Award
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http: //www.wjgnet.com

ISSN 1007-9327 CN 14-1219/R Local Post Offices Code No. 82-261

World Journal of Gastroenterology www.wjgnet.com Volume 12 Number 48 Dec 28 2006

ISSN 1007-9327
CN 14-1219/R

World Journal of Gastroenterology®

Indexed and Abstracted in:

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

Volume 12 Number 48
December 28, 2006

World J Gastroenterol
2006 December 28; 12(48): 7725-7888
Online Submissions
www.wjgnet.com/wjg/index.jsp
www.wjgnet.com
Printed on Acid-free Paper

A Weekly Journal of Gastroenterology and Hepatology

National Journal Award
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World Journal of Gastroenterology (*World J Gastroenterol , WJG*), a leading international journal in gastroenterology and hepatology, has an established reputation for publishing first class research on esophageal cancer, gastric cancer, liver cancer, viral hepatitis, colorectal cancer, and *H pylori* infection, providing a forum for both clinicians and scientists, and has been indexed and abstracted in Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®) and Journal Citation Reports/Science Edition, *Index Medicus*, MEDLINE and PubMed, Chemical Abstracts, EMBASE/Excerpta Medica, Abstracts Journals, *Nature Clinical Practice Gastroenterology and Hepatology*, CAB Abstracts and Global Health. ISI JCR 2003-2000 IF: 3.318, 2.532, 1.445 and 0.993. *WJG* is a weekly journal published by The *WJG* Press. The publication date is on 7th, 14th, 21st, and 28th every month. The *WJG* is supported by The National Natural Science Foundation of China, No. 30224801 and No. 30424812, which was founded with a name of *China National Journal of New Gastroenterology* on October 1,1995, and renamed as *WJG* on January 25, 1998.

PUBLISHED BY
The WJG Press

PRINTED BY
Printed in Beijing on acid-free paper by Beijing Kexin Printing House

 Print ISSN 1007-9327
CN 14-1219/R.

EDITORIAL OFFICE
World Journal of Gastroenterology,
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Institutional Rates
2006 rates: USD 1500.00

Personal Rates
2006 rates: USD 700.00

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PO Box 2345, Beijing 100023, China
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World J Gastroenterol 2006 December 28; 12(48): 7725-7736
World Journal of Gastroenterology ISSN 1007-9327

EDITORIAL

New insights into the coagulopathy of liver disease and liver transplantation

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Received: 2006-08-18 Accepted: 2006-10-10

Abstract

The liver is an essential player in the pathway of coagulation in both primary and secondary haemostasis. Only von Willebrand factor is not synthetised by the liver, thus liver failure is associated with impairment of coagulation. However, recently it has been shown that the delicate balance between pro and antithrombotic factors synthetised by the liver might be reset to a lower level in patients with chronic liver disease. Therefore, these patients might not be really anticoagulated in stable condition and bleeding may be caused only when additional factors, such as infections, supervene. Portal hypertension plays an important role in coagulopathy in liver disease, reducing the number of circulating platelets, but platelet function and secretion of thrombopoietin have been also shown to be impaired in patients with liver disease. Vitamin K deficiency may coexist, so that abnormal clotting factors are produced due to lack of gamma carboxylation. Moreover during liver failure, there is a reduced capacity to clear activated haemostatic proteins and protein inhibitor complexes from the circulation. Usually therapy for coagulation disorders in liver disease is needed only during bleeding or before invasive procedures. When end stage liver disease occurs, liver transplantation is the only treatment available, which can restore normal haemostasis, and correct genetic clotting defects, such as haemophilia or factor V Leiden mutation. During liver transplantation haemorrage may occur due to the pre-existing hypocoagulable state, the collateral circulation caused by portal hypertension and increased fibrinolysis which occurs during this surgery.

Key words: Coagulation; Cirrhosis; Liver transplantation; Bleeding

Senzolo M, Burra P, Cholongitas E, Burroughs AK. New insights into the coagulopathy of liver disease and liver transplantation. *World J Gastroenterol* 2006; 12(48): 7725-7736

http://www.wjgnet.com/1007-9327/12/7725.asp

INTRODUCTION

The liver plays several key roles in blood coagulation being involved in both primary and secondary hemostasis[1]. It is the site of synthesis of all coagulation factors and their inhibitors except for von Willebrand factor (vWf)[2]. Liver damage is commonly associated with impairment of coagulation, when liver reserve is poor. The hemostatic system is in a delicate balance between prothrombotic and antithrombotic processes, aiming to prevent excessive blood loss from injured vessels and to prevent spontaneous thrombosis. Liver failure is accompanied by multiple changes in the hemostatic system, because of reduced plasma levels of procoagulative and anticoagulative clotting factors synthesised by hepatocytes and sinusoidal cells[3]. Vitamin K deficiency may coexist, so that abnormal clotting factors are produced due to lack of gamma carboxylation. Moreover during liver failure, there is a reduced capacity to clear activated hemostatic proteins and protein inhibitor complexes from the circulation. Thus the global effect of liver disease with regard to hemostasis is complex, so that patients with advanced liver disease can experience severe bleeding or even thrombotic complications (Table 1). Finally, when marked portal hypertension develops with collateral circulation and secondary splenomegaly, thrombocytopenia develops due to splenic sequestration. However, thrombocytopenia may also be due to decreased hepatic thrombopoietin synthesis. There is also impaired platelet function. These hemostatic abnormalities do not always lead to spontaneous bleeding, but the onset of complications of cirrhosis such as variceal bleeding or infection/sepsis may lead to worsening of the coagulation status. The presence of a consumptive coagulopathy other than secondary to sepsis or other predisposing causes is disputed.

Usually therapy for coagulation disorders in liver disease is needed only during bleeding or before invasive procedures. When end stage liver disease occurs, liver

transplantation is the only treatment available, which can restore normal hemostasis, and correct genetic clotting defects, such as hemophilia or factor V Leiden mutation. During liver transplantation hemorrage may occur due to the pre-existing hypocoagulable state, the collateral circulation caused by portal hypertension and increased fibrinolysis which occurs during this surgery.

HEMOSTATIC FACTORS

Procoagulant factors

The liver is the site of synthesis of fibrinogen and factors Ⅱ, Ⅴ, Ⅶ, Ⅸ, Ⅹ, Ⅺ and Ⅻ[4]. Von Willebrand factor (vWf) is synthetised by the endothelium[5]. Factor Ⅷ is synthetised mainly by the hepatic, but also non hepatic sinusoidal endothelial cells[6-8], thus the plasma concentration of factor Ⅷ is not decreased with liver disease, and may be even increased, as many chronic liver diseases are associated with chronic inflammation[9]. Factor Ⅷ is high in fulminant hepatic failure and low in disseminated intravascular coagulation (DIC)[10] but this differential diagnosis is seldom an issue in clinical practice.

Vitamin K is an essential cofactor for the production of biologically active forms of the coagulation factors Ⅱ, Ⅶ, Ⅸ and Ⅹ. When γ-carboxylation is impaired due to deficiency or antagonism of vitamin K, inert precurcors are synthesised, (known as Proteins Induced by Vitamin K Absence [PIVKA]) and released into the blood stream[11]. The clinical significance of these precursors is not clear. In the case of prothrombin, a specific and sensitive immunoassay for this incomplete PIVKA prothrombin detects changes before conventional coagulation tests[12]. In cholestasis, vitamin K absorption from the small intestine is reduced due to decreased bile salt production. It can be corrected by vitamin K 10 mg daily for 24-48 h, but in parenchymal liver disease as there is a decreased synthesis of coagulation factors, there is no improvement with vitamin K[13]. However, 25% of patients with acute liver injury have a subclinical deficit of vitamin K which improves with parenteral administration of vit K[14].

In acute liver failure, plasma concentration of coagulation factors first those with the shortest half life, factor Ⅴ and Ⅶ (12 h and 4-6 h respectively), and factors Ⅱ, Ⅶ and Ⅹ subsequently[15]. Factor Ⅷ, together with vWf is usually elevated. The differential effects on clotting factor concentrations during acute liver failure occur because high cytokine concentrations increasing tissue factor (TF) which activates factors Ⅱ, Ⅴ, Ⅶ, Ⅹ, whereas any thrombin generated is inhibited by antithrombin Ⅲ, preventing activation of factors Ⅷ, Ⅺ and consequently Ⅺ, thus preserving their plasma levels[9].

Prothrombin gene mutation (G20210A) is the most common thrombophilic cause of portal vein thrombosis without cirrhosis (22% of cases)[16]. In contrast, factor Ⅴ Leiden mutation is common thrombophilic disorder (20%) associated with hepatic vein thrombosis in Western countries[17].

vWf

Plasma concentration of vWf is increased in patients with acute liver failure, due to increased synthesis as an acute phase protein in response to tissue injury[18-20] and also endothelial dysfunction secondary to endotoxemia[5]. In chronic liver disease, endothelial shear stress related to portal hypertension may also contribute to the high plasma levels of vWf *via* a nitric oxide stimulus[21]. A correlation between severity of liver disease and vWf plasma antigen levels has been documented.

Fibrinogen

Plasma fibrinogen is an acute-phase reactant, and remains normal or increased in patients with liver disease[22]. Low concentrations due to decreased synthesis, yet above 100 mg/dL, are only seen with very severe liver disease[23]. However the high fibrinogen concentrations found in patients with chronic hepatitis, cholestatic jaundice and hepatocellular carcinoma, do not result in increased clot formation as most is a non-functional fibrinogen present in 60%-70%: there are abnormal α chains and a higher sialic acid content[24]. This is due to an increased activity of sialil-transferase in immature hepatocytes generated during hepatic injury; this results in an abnormal thrombin time (TT), despite an almost normal PT and PTT, with an apparent normal or raised concentration of fibrinogen.

Platelets

Abnormalities in both number and function of platelets are common in liver disease and contribute to the impaired hemostasis.

About one third of patients with chronic liver disease develop thrombocytopenia, (70.000-90.000 × 10^9/L), which worsens in parallel with disease progression associated with increased platelet sequestration due to hypersplenism[25-27].

Thrombocytopenia appears not to be associated with an increased risk of bleeding from esophageal varices or other sites, although there are only few studies evaluating this, but it is correlated with blood loss during surgery[28]. A higher spleen diameter/platelet count ratio is highly predictive for the presence of esophageal varices in patients with liver cirrhosis[29].

Splenic sequestration versus other causes of thrombocytopenia in cirrhosis has been recently evaluated by comparing platelet number in extrahepatic portal hypertension, to that of cirrhosis in patients having a similar sized spleen. There is less severe thrombocytopenia in the non-cirrhotic patient[30]. Synthetic function of the liver is essential for platelet production *via* thrombopoietin (TPO), which regulates platelet production in the bone marrow[31]. Although TPO increases in patients with thrombocytopenia due to a homeostatic response[32], this occurs to a lesser degree with severe or chronic liver disease, than in patients with a normal liver[33]. Lower TPO mRNA levels in cirrhotic liver tissue[34] have been shown, confirming impaired TPO synthesis. In addition, a low platelet production from the bone marrow in cirrhotic patients has been shown[35].

Hepatitis C virus (HCV)[36] acute viral infection, alcohol abuse and folate deficiency can all result in some myelosuppression[37] further lowering platelet counts. Thrombocytopenia may also be contributed to by immune mediated mechanisms due to an increase production from B cells of antibodies binding platelet surface antigen GPI-

Table 1 Hemostatic abnormalities associated with liver disease

Favoring hemorrage	Favoring thrombosis
Low platelet count	
Impaired platelet function and platelet-vessel wall interaction	Elevated levels of factors Ⅷ and vWf
Enhanced platelet inhibition by nitric oxide (NO) and prostacyclin	Decreased levels of protein C, protein S, antithrormbin
Ⅲ, α_2-antiplasmin	
Decreased levels coagulation factors (Ⅱ, Ⅴ, Ⅶ, Ⅸ, Ⅹ, Ⅺ)	Macroglobulin
Quantitative and qualitative abnormalities of fibrinogen	Heparin cofactor Ⅱ elevated
Low level of α_2-antiplasmin, TAFI, histidine-rich-glycoprotein	Decreased levels of plasminogen
levels of tPA, with small increase of PAI-1 levels	

Ib-Ⅲa and GPIb/I, shown in viral related cirrhosis B and C[38], primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC)[39].

Platelet aggregation in response to ADP, arachidonic acid, collagen and thrombin is subnormal, probably due to a defective signal transduction mechanism[19]. Intrinsic defects including an abnormal arachidonic acid membrane content[40] and abnormal plasma factors[41] have also been shown to contribute to platelet function abnormalities. In cholestatic liver diseases there often is a normal or hypercoagulable state evaluated by thromboelastography[42] and there can be normal or hyperactive platelet function when assessed by platelet function assay (PFA-100) closure time and flow cytometric study of receptors[43]. When platelet number is too low, both cytometry and aggregation studies may be difficult to interpret. Thromboelastography which is a global test of clot formation and dissolution measures both platelet function and number by the maximum amplitude (ma) parameter[44] which can be used to assess platelet function.

Splenectomy is generally contra-indicated in patients with liver cirrhosis, because of the high mortality rate and a risk of secondary portal vein thrombosis , which leads to bleeding from esophageal-gastric varices and more difficult surgery during subsequent liver transplant[45]. Splenic embolization with 30%-50% reduction in flow can normalize or significantly improve platelet number in some cirrhotics[46] and it is sometimes used before embolisation of hepatocellular carinoma or interferon therapy for viral hepatitis. Insertion of transjugular intrahepatic portosystemic shunt (TIPS) increases, but it does not to normalize platelet number[47,48].

ANTICOAGULANT FACTORS

Antithrombin Ⅲ

Antithrombin Ⅲ (ATⅢ) is a non-vitamin K-dependent glycoprotein synthesised by the liver and endothelium[49]. In liver diseases, concentration falls due to reduced synthesis and/or increased consumption due to hyperfibrinolysis[50]. Usually the ATⅢ deficit is mild and thrombotic complications are very rare[51]. ATⅢ replacement does not correct hyperfibrinolysis in cirrhotic patients.

Protein C and protein S

Proteins C and S are vitamin K dependent glycoproteins synthesised mainly by hepatocytes[52]. During acute or chronic liver disease, their concentrations decrease concomitantly with the other coagulation factors, but usually not below 20% of normal[53]. Genetic deficiency of protein C is rare in the general population and portal vein thrombosis[54], but is found in 20% patients with Budd-Chiari syndrome (BCS). In patients with liver disease who also have genetic deficiency, plasma concentration is often lower than 20%. When there is severe liver disease, it can be difficult to exclude coexistent genetic deficiency as levels may be very low, due to very depressed synthesis[17]. In this situation a concomitant finding of a normal level of factor Ⅱ and protein C/factor Ⅶ ratio, can help to confirm a coexistent genetic deficit[55]. Genetic deficiency of protein S is extremely rare, but accounts for 7% of patients with BCS or portal vein thrombosis (PVT), especially in series from Asia[56].

DISORDERS OF THE FIBRINOLYTIC SYSTEM

All the proteins involved in fibrinolysis, except for tPA and PAI-1 are synthesized in the liver. Reduced plasma levels of plasminogen[57], α2-antiplasmin, histidine-rich glycoprotein (HRG)[58], factor XⅢ[59], and thrombin-activable fibrinolysis inhibitor (TAFI)[57] are found in cirrhosis. Conversely tPA levels are increased in liver disease, due to decreased clearance, whereas it's inhibitor PAI-1 is normal or only slightly increased in plasma. The inhibitor concentrations are insufficient to counteract the increase in tPA, accounting for increased fibrinolysis[60]. In contrast, in acute liver failure, there are high levels of the acute phase reactant PAI-1 leading to a shift towards hypofibrinolysis[61].

Hyperfibrinolysis is correlated with the severity of liver dysfunction in cirrhosis as assessed by Child-Pugh score[62]. Ascitic fluid has increased fibrinolytic activity: up to 20 liters are reabsorbed daily, with fibrinolysis being correlated with endotoxin levels[63]. Increased levels of D-dimers, prothrombin fragments 1+2 (F1+2) fibrin degradation products and plasmin-α2-antiplasmin complexes are found[64]. Many studies using different methodologies demonstrate hyperfibrinolysis (thromboelastography[65], diluted whole blood clot lysis assay[66] and euglobin clot lysis time[67]) . TAFI is decreased by an average of 26% in cirrhosis and by 50% in acute liver failure[68,69]. However there is some controversy as regarding hyperfibrinolytic activity in cirrhotics as not all studies have confirmed this.

Interestingly, patients with cholestatic liver diseases, are characterized by a normal or hypercoagulable state: higher PAI-1 concentrations are seen compared to other etiologies, balancing the increased tPA activity. This results in less hyperfibrinolysis in the reperfusion phase during liver transplantation, and antifibrinolytic therapy is not usually administered[70]. Thus the clinical issue is whether cirrhotic patients when under "stress" (e.g. during infection, during surgery or during bleeding) exhibit the increased fibrinolysis, resulting in an increased bleeding tendency, which is not manifest in laboratory terms when patients are stable.

Disseminated DIC and accelerated intravascular coagulation (AIC)

DIC is characterized by intravascular fibrin deposition due to activation of the clotting cascade, which overwhelms the anticoagulation pathway. Secondarily there is consumption of coagulation factors and platelets, associated with secondary fibrinolysis, causing an increased bleeding tendency[71].

Low grade DIC and the hemostatic abnormalities which are present in cirrhotics; they share common laboratory features, ie a prolonged PT and PTT, low fibrinogen level, elevated fibrin-degradation product and D-dimer and thrombocytopenia[72-74]. Thus differential diagnosis by laboratory means alone may be confounding. Early reports linked chronic liver disease to low grade DIC, ascribing the latter to accelerated fibrinolysis. However, the presence of DIC in liver cirrhosis is disputed[75]. Although DIC-like laboratory abnormalities (so called "pseudo-DIC) are observed, autopsy studies in cirrhotics have shown little evidence for fibrin deposition and clinically manifest DIC is very rare[72].

More highly sensitive tests such as quantification of proteolytic cleavage products of the coagulation reaction ie fibrinopeptide A, F1+2, and fibrinolysis reactions (fibrin D-dimer, high molecular weight fibrin/fibrinogen complexes or soluble fibrin), demonstrate an abnormal profile called accelerated intravascular coagulation and fibrinolysis phenomenon (AICF)[75]. The studies to date demonstrate AICF in about 30% of cirrhotics, depending on the severity of liver disease[65].

However, Ben Ari *et al* analyzed 52 patients with stable liver disease for F1+2 thrombin-antithrombin Ⅲ complex (TAT) and D-dimer levels which were no different from controls, yet TEG studies were able to detect hyperfibrinolysis. AICF may be important in the portal venous system, as this phenomenon is more pronounced there than in systemic blood[65]. This could be related to higher levels of endotoxemia in portal blood, which can trigger release of IL6 and TNF-alfa thus activating intravascular coagulation[76].

PROGNOSTIC VALUE OF COAGULATION FACTORS

In cirrhosis, plasma levels coagulation factors are indicators of hepatic synthesis and thus of liver function. A prolonged PT, which is not corrected by intravenous vitamin K administration 10 mg daily for 2 d, helps differentiate vitamin K deficiency from parenchymal liver diseases[13]. PT is part of the Child-Pugh score, which is the most commonly used prognostic score assessing the severity of liver disease[77]. Recently the MELD score which incorporates INR has been used to allocate priority for liver transplantation in the USA based on estimated probability of death within 3 mo[78].

Determination of individual coagulation factors adds little prognostic information to measuring PT or INR in cirrhosis. A multivariate analysis of prognostic factors in cirrhotic patients showed that the level of factor Ⅶ was an independent predictor factor of survival: factor Ⅶ < 34% was predictive of a mortality in 93%[79].

In acute liver failure, the Clichy criteria indicate poor prognosis and need for liver transplantation, when factor V is below 20% in patients aged ⩽ 30 or below 30% associated with age ⩾ 30[80]. Factor V has less prognostic value in acetaminophen-induced fulminant hepatic failure[81].

In the King's College criteria in acetaminophen-induced liver failure, PT ⩾ 100 s is a prognostic indicator on its own for liver transplantation independent of the grade of coma. In patients with non-acetaminophen induced ALF, PT ⩾ 50 s together with two of the following criteria: age < 10 > 40 years, drug toxicity, interval between jaundice and encephalopathy onset > 7 d and serum biliubin > 300 μmol/L are indications of poor prognosis and for liver transplantation[82].

ASSESSMENT OF THE RISK OF THROMBOSIS AND ANTICOAGULATION

Thrombotic complications can paradoxically occur in cirrhotic patients even if clinically an increased risk of haemorrhage is considered. Despite prolonged coagulation tests, these patients cannot be viewed as being "anti-coagulated". Wanless *et al* has put forward portal and hepatic vein thrombosis as cause of disease progression in cirrhotic patients. Hepatic and portal vein thrombosis was found in at least 70% of explanted livers, and 36% were associated with regions of confluent fibrosis (focal parenchymal extinction)[83], which is a histological correlate of chronic thrombosis.

Portal vein thrombosis complicates liver cirrhosis between 0.6% to 15% of cases, leading to worsening of liver function, development of ascites and occasionally mesenteric infarction[84]. In these patients early anticoagulation is indicated and has been shown to recanalise the splanchnic veins in about 50% of cases and prevent the extension of the thrombus without causing increased haemorragic complications[85].

In BCS, even if a prothrombotic cause is not identified, anticoagulation should be started immediately after diagnosis, as many genetic prothrombic defects remain yet to be identified and acquired disorders, common in BCS, may be difficult to diagnose, such as polycythaemia rubra vera or paroxysmal nocturnal hemoglobinuria (PNH). Early anticoagulation ameliorates prognosis. Anticoagulation therapy should continue even after liver

transplantation because of the high rate of recurrence and thrombotic complications after OLT, and also because other prothrombotic disorders may exist alongside the diagnosed protein deficiencies[17,86].

The risk of deep vein thrombosis and pulmonary embolism is not well documented in cirrhotics, yet is reported[87]. Patients with cholestatic disease often exhibit a procoagulant state demonstrated by TEG, may be prone thrombosis, but this has not been studied[42]. No guidelines are available for the management of thrombotic complications and neither for prevention of embolic phenomena for example following atrial fibrillation in cirrhotic patients.

ASSESSMENT OF THE RISK OF BLEEDING

The role played by coagulation defects in the occurrence of bleeding in cirrhosis is still unclear. This is particularly due to the difficulty (and cost) in measuring procoagulant and anticoagulant activities, and assessing the balance between the two (Table 1). In addition there are very few tests which reflect coagulation *in vivo*. Recently generation of thrombin has been explored *in vitro* in cirrhotic patients and found to be normal. In this study, a resetting of the coagulation and anticoagulation system at a lower level was postulated, because during liver disease both procoagulant and anticoagulant pathways are affected in a parallel manner. However, the in vitro technique has some drawbacks, the major one being that platelets are substituted by phospholipids[3].

Minor signs of bleeding tendency are common, such as gum bleeding and epistaxis, but major bleeding can be encountered. The role of hemostatic abnormalities in variceal bleeding is not clear. Hyperfibrinolysis has been shown to be linked but not necessary causal to an increased risk of variceal bleeding, in a cohort of 61 cirrhotics. Higher levels of fibrinogen degradation products were associated with a greater risk of variceal bleeding compared to patients without (odds ratio = 8), but Child-Pugh score and endoscopic characteristics of varices remain the most important prognostic factors[88]. Recently the role of infection and endogenous heparin-like substances demonstrated by TEG has been evaluated in variceal bleeding. Infection may be a trigger factor for bleeding[89] and both infection and heparin-like substances may be mechanisms responsible for the persistence of bleeding in some[90]. TEG, which is a quick and reliable method to assess clot formation and lysis[44], also allows detection of heparin-like substances. Studies from our group have shown worsening coagulation during infection due to low molecular weight heparin-like substances detected by TEG[91].

INVASIVE PROCEDURES

Historically, PT and platelet count have been used to assess the risk of bleeding prior to invasive procedures. Cirrhotic patients have increased mortality and morbidity during surgery[92], mainly due to increased bleeding in 60% of cases[93,94]. Early studies linked PT to surgical risk (PT prolongation > 1.5 and > 2.5 s associated with 47% and 87% mortality respectively)[95], hence platelet count < 50.000/mm^3 and PT > 3 s have been considered relative contraindications to elective surgery[94]. In addition, portal hypertension and collateral veins increase the risk of bleeding during surgical dissection.

Hyperfibrinolysis[96] and clotting activation, due to increase tPA levels have been described in patients undergoing liver resection[97]. However, another study performed in patients undergoing laparoscopic liver biopsy failed to demonstrate any correlation between the risk of bleeding evaluated at the hepatic puncture site and coagulation tests, so that the degree of injury may be the important factor[98].

Liver biopsy is widely used diagnostically and to grade the severity of liver disease or fibrosis. Moreover it is an essential tool after liver transplantation to diagnose rejection and other causes of graft dysfunction. Bleeding complications occur in 0.35%-0.5%, leading to mortality in 0.1%[99]. Despite the evidence that there were no threshold abnormalities of clotting tests associated with risk of bleeding during laparoscopic liver biopsy, INR and platelet count are considered essential to evaluate the bleeding risk for percutaneous liver biopsy[100]. An audit from the British Society of Gastroenterology (BSG) performed in 1991 showed a doubling of bleeding risk in patients with INR $\geqslant$ 1.5, but that only 7.1% of the bleeding occurred with INR greater than 1.5, and 90% occurred with a INR $\leqslant$ 1.3[101]. A cut off for platelet count is difficult to justify from the literature. Most textbooks in the UK and BSG guidelines, require platelet count above 80.000/mm^3[13] whereas a survey form the Mayo Clinic suggested 50.000/ mm^3 as a cut off[102]. Current recommendations state that a percutaneous liver biopsy can be done safely without support with platelet counts are above 60.000/mm^3[100]. Burroughs *et al* advocated evaluating the use of bleeding time to assess the risk of bleeding for percutaneous liver biopsy[103], but this is not routine in clinical practice. If clotting parameters are outside stipulated ranges, a transjugular liver biopsy can be performed more safely, without plasma or platelet therapy[104]. A plugged liver biopsy is also said to be safer, but it may cause greater risk of bleeding in hypocoagulable patients[99].

During minor procedures such as thoracentesis, paracentesis or lumbar puncture performed in patients with liver disease, there are no firm guidelines as to the hemostatic threshold for performing these tests. A contraindication to the procedure is clinically evident DIC or fibrinolysis[105].

COAGULATION DURING INFECTION AND SEPSIS

The overall cumulative incidence of infection in cirrhotic patients is estimated to be at least 30%[106], and is possibly associated with increased risk of variceal bleeding[89]. Infection is associated with early rebleeding and increased mortality[107,108]. Prophylactic antibiotic therapy has led to less early rebleeding and better control of bleeding, in a randomized study[109].

Using TEG, 20 cirrhotic patients who experienced early

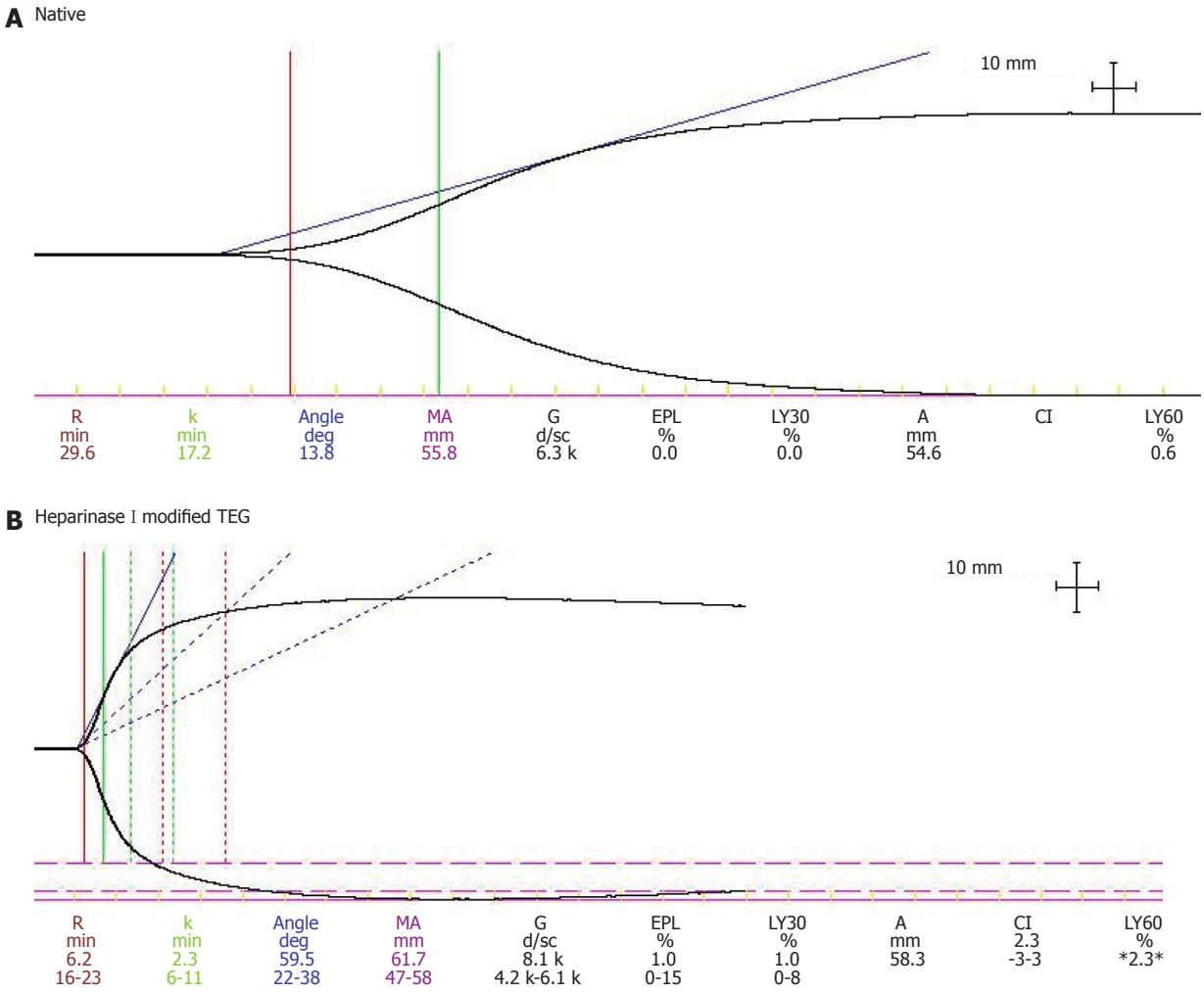

Figure 1 Native-TEG (**A**) and heparinase I -TEG (**B**) on sample collected at the onset of spontaneous bacterial peritonitis in a patient with liver liver cirrhosis. (**A**) significant heparin-like effect found revealed by the slowed rate of coagulation. (**B**) treatment of the sample with heparinase I increases the rate of coagulation, thus sampling the presence of heparin-like substances.

rebleeding were found to have worsening TEG parameters the day before rebleeding[90], Moreover patients with bacterial infection have worse TEG parameters, which are corrected *in vitro* by heparinase I, which cleaves heparin-like substances[91] (Figure 1A and 1B). The presence of heparin like substances is associated in some with increased antiXa activity[110]. Heparin-like substances have been detected hours after variceal bleeding in cirrhotic patients[111]. Based on this evidence the hypothesis has been postulated that endotoxins and inflammation due to infection can release heparinoids from the endothelium and mast cells[91]. Moreover sepsis can cause impairment of platelet function, decreasing platelet number and aggregability, due to increase NO production[112].

THERAPY OF HEMOSTATIC ABNORMALITIES IN LIVER DISEASE

Therapy for hemostatic abnormalities of liver disease is needed only during variceal bleeding, surgery or before invasive procedures. Intravenous vitamin K injecton of 10 mg daily for 24-48 h can replace vitamin K deficiency[13].

Fresh frozen plasma (FFP) contains all the clotting factors and can correct the laboratory finding of an elevated PT effectively, but this correction depends on the volume and the baseline abnormality of PT. Whether this correction of the PT results in increasing hemostasis has yet to be proven. In addition, correction is short term (24-48 h), depending on the half-life of the clotting factors (especially factor Ⅶ)[71]. A common indication for FFP infusion is the presence of persistent bleeding in patients with INR $\geqslant$ 2 or PT prolongation greater than 4 s[113]. In surgical or invasive procedures 50% of the normal PT (ie INR of 2) is a target for replacement therapy, and for neurological procedures such as intracranial pressure monitoring during liver failure, 80% of normal PT range (ie an INR of about 1.2-1.3)[113]. During massive blood transfusion, to avoid dilutional decrease of clotting factors for every 2 units of blood, 1 of FFP is typically given[114]. To increase the activity of clotting factor by 1%-2% a dose of 1 mL FFP/kg of body weight is necessary[115]. Because of the high volume required, adequate replacement is difficult both in cirrhotic patients (intravascular plasma volume is already expanded and ascites may be present), and ALF, (increasing plasma volume can lead to increases in intracerebral pressure). Moreover, the short half-life requires infusion every 6-12 h[10]. In patients with INR > 1.5,

FFP is given (12-15 mL/kg) before liver biopsy, but there is no evidence base for this. Transjugular biopsy should be used in patients with coagulopathy not sufficiently corrected with FFP.

Platelet transfusion, one unit every 10 kg is typically administered, and platelet count should be checked 1 h after the infusion[116]. However no correlation between amelioration of bleeding time, increase in platelet count, and enhanced hemostasis has been shown[100].

Cryoprecipitate contains factors Ⅷ, fibrinogen, vWf, fibronectin and XIII. Because of the small volumes (30-50 mL/U/10 kg) required[116], it can be useful in liver cirrhosis and ALF, but it lacks some coagulation factors and may worsen the imbalance already present in patients with liver disease.

Desmopressin (1-deamino-8-D-arginine vasopressin [DDAVP]), an analogue of the antidiuretic hormone, increases plasma level of factor Ⅷ and vWf, probably by increasing the release from endothelial storage sites[117]. It can improve bleeding time, enhancing primary hemostasis at the dose of 0.3 μg/kg in patients with liver failure[118]. However a randomised trial associating terlipressin and DDAVP in patients with variceal bleeding, demonstrated no difference in control of bleeding and maybe a worsening of the terlipressin action in the DDVAP group[119]. In a recent randomized trial, DDVAP failed to decrease blood loss during hepatic resection, despite increase of factor Ⅷ and vWf[120]. ATⅢ infusion is not routinely recommended.

Recombinant activated factor Ⅶ (rFⅦa) was first developed for the treatment of patients with hemophilia A and B who developed inhibitors. It may have promising role in the treatment of coagulation disorders in liver disease[121]. A single dose of recombinant factor Ⅶ a has been shown to correct prolonged PT in a dose-dependent manner in non-bleeding cirrhotic patients[122]. A randomized study using rFⅦa in 71 patients undergoing laparoscopic liver biopsy found no differences in liver bleeding time. Two complications occured in the rFⅦ a group (1 DIC and 1 PVT)[123]. In ALF, rFⅦa may be useful to normalize PT in the setting of intracranial pressure monitoring, as only a small volume of infusion is required. During variceal bleeding in a randomized trial, a modest reduction of early rebleeding rate was observed in a subgroup of Child B and C patients after rFⅦa infusion, although, no difference in control of bleeding or transfusion was shown overall[124]. Another report described initial hemostasis after infusion of rFⅦ a in 10 patients with variceal bleeding, but 6 experienced early rebleeding and all of them died, illustrating the short interval of action of this drug[125]. In a cohort of 8 patients with acute variceal bleeding uncontrolled with endoscopic and medical therapy, rFⅦa administration achieved hemostasis in 25% after a single dose[126].

Safety of rFⅦa, especially about the possible prothrombotic effect or triggering of DIC, still has to be assessed in large studies in patients with liver disease[127].

LIVER TRANSPLANTATION

Orthotopic liver transplantation (OLT) is the only cure for end stage liver disease. Improvements in operative management, surgical techniques and graft preservation have contributed to a significant reduction in transfusion requirements during the last decade[128]. However, blood losses are highly variable, and correlate in most studies with a higher mortality, poor graft function and risk of infections[129]. In current practice a significant proportion of patients receive no blood during surgery.

Most studies failed to define factors related to bleeding, including preoperative coagulation tests or markers of fibrinolysis during liver transplantation[130-132], with the exception of the collateral circulation due to portal hypertension and previous abdominal surgery [133].

Hemostatic abnormalities during liver transplantation are divided according to the surgical phases which are traditionally: pre-anhepatic phase, anhepatic phase and post reperfusion phase and post operative period.

Pre-anhepatic stage

The first operative stage is characterized by extensive surgical trauma, resulting from dissection of adhesions in the abdominal cavity and transection of many collateral vessels. Usually during this phase, mild coagulation abnormalities occur and the blood losses are mainly correlated with the surgical technique and the baseline hypocoagulable state[133], but etiology of liver disease can also influence the blood product requirement. Hypercoagulability has been demonstrated in patients with hepatocellular carcinoma as well as cholestatic cirrhosis (PBC, PSC). The PBC and PSC patients have a hypercoagulable state by TEG[42] and less fibrinolytic activity during OLT than other aetiologies[134], suggesting that in these patients antifibrinolytic drugs should not be used. Moreover in pediatric liver transplantation for biliary atresia, plasma studies showed less coagulation abnormalities during OLT compared to other etiologies[135]. Enhanced fibrinolytic activity contributes to blood loss in the pre-anhepatic phase in only 10%-20% of patients[136].

Anhepatic phase

During this phase no important surgical blood loss is seen because appropriate vessels are clamped. However, bleeding can occur due to hemostatic changes in this phase. Despite impairment of synthetic and clearance function, early studies failed to show dramatic changes in PT and PTT[129,137]. However, hyperfibrinolysis has been demonstrated in many studies, due to net increase in tPA derived from endothelial cells; this tPA is not cleared due to the absence of the liver at this time[138]. The presence of an active fibrinolytic process has been demonstrated by simultaneous decrease of α2-antiplasmin and plasminogen activity, and a concomitant increase in fibrin and fibrinogen degradation products[139]. Use of rFⅦa has been tried in patients with severe coagulopathy (INR 5.7 and 6.9). Moderate bleeding was still reported during surgery, but 1 patient developed hepatic artery thrombosis after transplant[140]. Studies which evaluated coagulation factors during OLT after rFⅦa infusion showed a sharp increase of thrombin generation, PT and PTT, but no amelioration of fibrinolysis[141,142].

Reperfusion and post reperfusion phase

Reperfusion of the liver is a crucial point of the operation and leads to profound coagulation abnormalities. Within minutes after reperfusion, uncontrollable diffuse bleeding may occur in some patients[143].

Trapping of platelets in the graft may play a role in the bleeding tendency. Experimental studies have shown a 55% gradient in platelet count between arterial and venous blood flow in the new liver. Moreover, some alteration in the bleeding time and platelet function and aggregation have been demonstrated[144]. Signs of DIC after graft reperfusion have been shown by some investigators, mainly correlated with poor quality of the transplanted organ[145].

Increase in fibrinolysis has been implicated as the most important and significant phenomenon responsible for bleeding during liver transplantation. It usually subsides within 60 min after graft reperfusion, but in donor livers with poor function, a sustained increased fibrinolytic response can be seen[146].

After reperfusion, release of heparin or heparin-like substances has been shown in 25%-95% of cases[147]. Protamine sulphate (50 mg) has been used *in vivo* to antagonize this effect. One study has confirmed the presence of heparin-like compounds using heparinase I-modified TEG, which cleaves heparin and heparan sulphate. Increased blood product requirement was correlated with the presence of heparin like effects in TEG traces. However a baseline heparin-like effect has recently been found before reperfusion in patients undergoing liver transplantation not receiving heparin[148].

Antifibrinolytic therapy is used during liver transplantation to reduce blood loss, time of surgery and fibrinolytic activity. Aprotinin is a serine protease inhibitor which antagonizes various proteases[149]. Aprotinin also has anti-inflammatory and anti-oxidant effects which might also be of benefit. Widespread use of aprotinin is not recommended because of the risk of anaphylactic reactions, renal dysfunction and stroke[150], which has been also recently stressed by a multicentre study on 4357 patients undergoing cardiac surgery[151], but this is not been reported nor studied in liver transplantation.

Epsilon aminocaproic acid (EACA) interferes with plasminogen binding to fibrin and thus EACA inhibits the conversion of plasminogen to plasmin[152]. In the only prospective randomized trial, it was shown to reverse TEG fibrinolysis, and reduce blood cell transfusion, without causing thrombotic complications. However this reduction was not statistically significant compared to controls[153]. Similar to EACA, tranexamic acid inhibits fibrinolysis, but it is 6 to 10 times more potent than EACA[154]. Recent trials have shown that at a dose of 2 mg/kg per hour, tranexamic acid reduces fibrinolysis and blood loss. However different doses have been used in other studies without clearcut effects[155-157].

The routine use of coagulation monitoring during liver transplantation is common place. Usually TEG is used, a point of care rapid method to assess the whole coagulation process. It provides the basis of a rational approach to the use of blood component therapy or pharmacologic intervention, but it does not help in addressing blood transfusion per se[44]. Recently TEG was used to monitor postoperative coagulation in patients undergoing hepatic resection for living related liver transplantation. In these patients, a hypercoagulable state correlated with the risk of developing thrombotic complications after surgery[158].

Postoperative period

Thrombocytopenia is common in the early post-operative period, mainly due to platelet activation and consumption following graft reperfusion[159]. Thrombocytopenia is common in the early post-operative period, mainly due to platelet activation and consumption following graft reperfusion[159], and if liver function restores thrombocytopenia subsides a few day after OLT. Following normal synthetic function of the liver, thrombopoietin levels increase significantly on the first day, following by immature bone marrow megakariocites after 3 d and new circulating platelets after 5 d Normalization of platelet count can be seen after 14 d[160]. Peak of TPO level correlates with the pre-OLT platelet count. Levels of bilirubin, cold ischemia time or episodes of rejection do not influence TPO levels[161]. Persistence of thrombocytopenia can be seen in some patients, which can be ascribed to persistent splenomegaly in some[162].

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S- Editor Wang J **L- Editor** Chiarioni G **E- Editor** Bai SH

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World J Gastroenterol 2006 December 28; 12(48): 7737-7743
World Journal of Gastroenterology ISSN 1007-9327

EDITORIAL

Contribution of altered signal transduction associated to glutamate receptors in brain to the neurological alterations of hepatic encephalopathy

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Supported by grants from the Ministerio de Ciencia y Tecnología, No. SAF2002-00851 and SAF2005-06089 and from Ministerio de Sanidad, No. Red G03-155 and PI050253 of Spain and by grants from Consellería de Empresa, Universidad y Ciencia, and de Sanidad, Generalitat Valenciana, No. Grupos03/001, GV04B-055, GV04B-012, GVS05/082 and ACOMP06/005 and AP-005/06
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Received: 2006-08-17 Accepted: 2006-09-19

Abstract

Patients with liver disease may present hepatic encephalopathy (HE), a complex neuropsychiatric syndrome covering a wide range of neurological alterations, including cognitive and motor disturbances. HE reduces the quality of life of the patients and is associated with poor prognosis. In the worse cases HE may lead to coma or death.

The mechanisms leading to HE which are not well known are being studied using animal models. The neurological alterations in HE are a consequence of impaired cerebral function mainly due to alterations in neurotransmission. We review here some studies indicating that alterations in neurotransmission associated to different types of glutamate receptors are responsible for some of the cognitive and motor alterations present in HE.

These studies show that the function of the signal transduction pathway glutamate-nitric oxide-cGMP associated to the NMDA type of glutamate receptors is impaired in brain *in vivo* in HE animal models as well as in brain of patients died of HE. Activation of NMDA receptors in brain activates this pathway and increases cGMP. In animal models of HE this increase in cGMP induced by activation of NMDA receptors is reduced, which is responsible for the impairment in learning ability in these animal models. Increasing cGMP by pharmacological means restores learning ability in rats with HE and may be a new therapeutic approach to improve cognitive function in patients with HE. However, it is necessary to previously assess the possible secondary effects.

Patients with HE may present psychomotor slowing, hypokinesia and bradykinesia. Animal models of HE also show hypolocomotion. It has been shown in rats with HE that hypolocomotion is due to excessive activation of metabotropic glutamate receptors (mGluRs) in substantia nigra pars reticulata. Blocking mGluR1 in this brain area normalizes motor activity in the rats, suggesting that a similar treatment for patients with HE could be useful to treat psychomotor slowing and hypokinesia. However, the possible secondary effects of mGluR1 antagonists should be previously evaluated.

These studies are setting the basis for designing therapeutic procedures to specifically treat the individual neurological alterations in patients with HE.

Key words: Hepatic encephalopathy; Glutamate receptors; Neurological alterations; Cognitive function; Motor function; NMDA receptors; Metabotropic glutamate receptors; Nitric oxide; cGMP

Felipo V. Contribution of altered signal transduction associated to glutamate receptors in brain to the neurological alterations of hepatic encephalopathy. *World J Gastroenterol* 2006; 12(48): 7737-7743

http://www.wjgnet.com/1007-9327/12/7737.asp

INTRODUCTION

Hepatic encephalopathy

Hepatic encephalopathy (HE) is a complex neuropsychiatric syndrome present in patients with chronic or acute liver disease. HE covers a wide range of neuropsychiatric disturbances ranging from minimal changes in personality or altered circadian rhythms (sleep-waking cycle) to alterations in intellectual function, personality, conscience and neuromuscular coordination. HE is usually reversible, but can lead to coma and death in the worse case.

The neurological alterations in HE are the result of a previous liver failure. Liver failure leads to impaired detoxification of ammonia and other toxic substances that can reach the brain and alter its function. Many studies have been carried out to identify factors responsible for

the neurological alterations in HE. Clinical experience and basic research indicate that ammonia is the main factor responsible for HE. Ammonia is a product of degradation of proteins and other nitrogenated compounds but at high concentrations ammonia is toxic, leading to alteration of cerebral function which can lead to coma and death.

Hyperammonemia is therefore considered the main factor contributing to the neurological alterations found in HE both in acute and in chronic liver disease[1]. Other factors may also contribute to neurological alterations in HE. It has been recently suggested that inflammation exacerbates the neuropsychological effects induced by hyperammonemia in cirrhosis[2].

Classical clinical treatment of HE is mainly directed to reducing ammonia concentration by reducing protein ingestion, lowering ammonia production by the intestinal bacteria as well as by reducing ammonia transport from intestine to the blood flow by acidification of the intestinal lumen.

Overt HE is usually elicited by one of the following precipitating factors (high protein ingestion, gastrointestinal constipation, bleeding, diuretics) usually associated with increased ammonia levels. HE is associated with a poor prognosis[3,4].

Liver cirrhosis patients with normal neurological and mental status may present minimal forms of HE, showing intellectual function impairment which cannot be detected through general clinical examination but can be unveiled using specific neuropsychological or neurophysiological examination[5,6]. Minimal hepatic encephalopathy (MHE) is the first stage in the spectrum of HE[7,8]. Patients suffering from MHE may present psychomotor slowing and cognitive deficits affecting their ability to perform certain activities in daily life. MHE is therefore associated with impaired quality-of-life[9,10]. Patients with MHE and altered oral glutamine challenge have a shortened life-span[7].

Treatment of MHE could prevent or delay the appearance of clinical HE and improve quality of life and life span of patients. In order to detect the presence of MHE and to treat it efficiently, it is necessary to know the mechanisms leading to MHE and subsequently to HE.

The mechanisms leading to HE are mainly studied using animal models of chronic liver failure and chronic hyperammonemia. The neurological alterations in HE are a consequence of impaired cerebral function mainly due to alterations in neurotransmission. Alterations in different neurotransmitter systems (glutamatergic, GABAergic, serotoninergic, *etc*) have been reported in HE[1].

We review here some studies indicating that alterations in neurotransmission associated to different types of glutamate receptors are responsible for some of the cognitive and motor alterations present in HE. Pharmacological manipulation of these receptors or pathways may normalize cognitive and motor function in animal models of HE.

INTELLECTUAL AND MOTOR FUNCTIONS ARE IMPAIRED IN HE

Cognitive, motor and sleep alterations (impairment of sleep-wake cycle) are commonly observed in patients with HE and their intensities vary with the grade of HE.

Alterations in the regulation of biological rhythms such as sleep, appetite, melatonin production and in sexuality are common in patients with liver disease[11-14].

Patients with HE show alterations in cognition, consciousness, attention, memory, and learning. Cirrhotic patients with minimal HE are "clinically normal" but present cognitive alterations which can be unveiled by a detailed analysis of the patients' history and by neurophysiological and neuropsychological assessment of consciousness and sensory, cognitive and motor functions[5,6]. The prevalence of minimal HE in patients with liver disease ranges from 30% to 84% depending on the kind and number of tests used and the population (etiology and severity of the liver disease) investigated. Even minimal HE is associated with reduced quality of life and ability to work and drive[9,15-17]. Moreover, patients suffering minimal HE have increased probability of suffering later overt HE[7,18,19].

Patients with minimal HE show impaired ability to perform memory tasks, mainly because of deficits in attention and visual perception[20,21]. These patients also perform worse than healthy controls in motor function, visual perception, visual orientation and visual-constructive abilities[22-25].

Sustained attention is also impaired in cirrhotic patients even when memory, language, or motor alterations are absent[21,26,27]. Patients with minimal HE have the tendency to be easily distracted.

Early manifestations of intellectual dysfunction in HE patients include psychomotor slowing and impaired ability to perform tasks that require sustained attention[18,26,28]. As encephalopathy worsens, impairment in speech and inability to copy simple drawings (e.g. a star) appear. Patients in grade Ⅱ HE show temporal and spatial disorientation and reduced vigilance state or delirium. Grade Ⅳ HE is characterized by the appearance of stupor and coma.

Patients with HE show also altered motor function and coordination, including psychomotor slowing and hypokinesia which was attributed to alterations in basal ganglia[29]. Motor alterations include increased muscular tone, reduced speed of rapid alternating movement, ataxia, an increased deep tendon reflexes, abnormal movements such as tremors, particularly asterixis. Also hypomimia, dysarthria, bradykinesia and hypokinesia could be detected on careful neurological examination.

The mechanisms by which liver failure leads to altered intellectual and motor function remain unclear. Identifying these mechanisms would allow designing treatments to improve intellectual and motor function in patients with HE.

Some motor and cognitive alterations present in HE patients are reproduced in animal models of chronic liver failure (e.g. rats with portacaval anastomosis) and chronic hyperammonemia[30-38]. These animal models are being used to study the molecular mechanisms by which liver disease leads to HE and altered intellectual and motor function. Once these mechanisms are identified, new studies begin

to assess whether similar alterations and mechanisms occur in brain of patients with HE.

GLUTAMATERGIC NEUROTRANSMISSION MODULATES COGNITIVE AND MOTOR FUNCTION AND IS ALTERED IN ANIMAL MODELS OF HE

Glutamate is the main excitatory neurotransmitter in mammals and modulates important cerebral processes including cognitive and motor functions (see below). Glutamatergic neurotransmission involves several steps, beginning with release of glutamate from the presynaptic neuron. Glutamate in the extracellular space activates glutamate receptors present in the synaptic membranes, leading to activation of signal transduction pathways associated to these receptors. To avoid continuous activation of glutamate receptors, glutamate is removed from the synaptic cleft by specific glutamate transporters located mainly in astrocytes. All these steps are tightly modulated under physiological conditions and alterations of any of the above steps may result in impairment of glutamatergic neurotransmission, leading to neurological alterations. Some of the parameters that can be altered under pathological conditions are: (1) the content (expression, synthesis and/or degradation) of the main proteins involved in glutamatergic neurotransmission (e.g. different types of glutamate receptors or transporters); (2) the regulation of the spatial location of the receptors and transporters. Only the receptors or transporters present in the membrane can recognize extracellular glutamate. Many of these proteins are associated to formation of clusters to improve the yield of the neurotransmission process; (3) the function of the receptors and transporters, which is modulated in different ways including phosphorylation-dephosphorylation, binding of co-agonists, *etc*; (4) alterations in the release or uptake of glutamate may result in altered extracellular glutamate, leading to altered neurotransmission; (5) alterations in any of the steps of the signal transduction pathways associated with the different types of glutamate receptors would also result in impaired glutamatergic neurotransmission.

There is therefore a large number of possible sites or molecular targets for interference by hyperammonemia or liver disease of glutamatergic neurotransmission. Hyperammonemia and liver failure alter different steps of glutamatergic neurotransmission including: glutamate concentration in the extracellular fluid in brain, transport and transporters of glutamate, content and function of different types of glutamate receptors and signal transduction pathways associated to these receptors[39].

Glutamatergic neurotransmission plays an important role in modulating intellectual function (learning and memory), motor function and coordination and circadian rhythms. As mentioned above, these processes are altered in patients with liver disease and HE, who show altered sleep-waking patterns, motor function and coordination and decreased intellectual capacity. The alterations in glutamatergic neurotransmission may be responsible for some of these neurological alterations found in HE patients.

We summarize below the alterations in glutamatergic neurotransmission that have been shown to contribute to the cognitive and motor alterations in hepatic encephalopathy.

Glutamate has two main types of receptors: ionotropic and metabotropic. Activation of ionotropic glutamate receptors leads to the opening of ion channels allowing the transport of Na^+, K^+ through them, and Ca^{2+} in some cases. There are three main types of ionotropic glutamate receptors: NMDA, AMPA and kainate receptors. The NMDA type of glutamate receptors is involved in the control of cerebral processes such as neuronal plasticity, learning and memory. Alterations in signal transduction pathways associated to NMDA receptors are involved in the impairment in cognitive function in HE (see below).

Metabotropic glutamate receptors (mGluRs) are coupled to G proteins. Activation of mGluRs modulates the activity of different enzymes (phospholipase C, adenylate cyclase, *etc*) and ion channels through these G proteins, resulting in modulation of the intracellular levels of second messengers such as diacylglycerol, inositol triphosphates, cAMP, *etc*. These second messengers in turn, modulate the activity of other enzymes (protein kinases C and A, *etc*) that continue the transmission of the signal induced by activation of metabotropic glutamate receptors.

Metabotropic glutamate receptors are involved in modulation of motor function. Alterations in activation of metabotropic glutamate receptors are involved in some motor alterations in HE (see below).

FUNCTION OF THE GLUTAMATE-NITRIC OXIDE-CGMP PATHWAY ASSOCIATED TO NMDA RECEPTORS IS ALTERED BOTH IN BRAIN *IN VIVO* ANIMAL MODELS OF HE AND IN BRAIN OF PATIENTS DIED FROM HE

Activation of NMDA receptors by glutamate leads to increased intracellular Ca^{2+} in the post-synaptic neurons. Ca^{2+} binds to calmodulin (CM) and activates different enzymes, including neuronal nitric oxide synthase (NOS), leading to increased formation of nitric oxide (NO), which in turn activates soluble guanylate cyclase (GC) and increases cGMP (Figure 1). Part of the cGMP formed is released to the extracellular space. Under appropriate conditions the increase in extracellular cGMP is a good measure of the extent of activation of NMDA receptors in brain *in vivo*.

The function of this glutamate-nitric oxide-cGMP pathway is impaired in brain *in vivo* in animal models of HE (rats with chronic hyperammonemia or with chronic liver failure). The most usual animal model for studying the neurological alterations in HE is the rat with chronic liver failure induced surgically by portacaval anastomosis. This model reproduces some of the neurological alterations found in patients with HE. Liver failure induces, in addition to hyperammonemia, other alterations (decreased muscle mass, altered metabolism of other compounds, *etc*).

To discern the contribution of hyperammonemia to the neurological alterations in HE, we developed an animal model of hyperammonemia without liver failure: rats fed an ammonium-containing diet[40,41]. These rats present a level of hyperammonemia similar to that of patients with liver cirrhosis or of rats with portacaval anastomosis, but do not present other alterations associated to liver failure and may be considered therefore as a model of "pure" hyperammonemia. Comparison of the effects induced by both models can clarify which effects are due to hyperammonemia and which are due to other factors associated to liver failure.

Using this model we found that chronic moderate hyperammonemia without liver failure impairs the function of the glutamate-NO-cGMP pathway in cerebellum *in vivo*, as shown by brain microdialysis in freely moving rats[42]. When microdialysis probes are inserted in the cerebellum of control or hyperammonemic rats without liver failure, administration of NMDA through the microdialysis probe activates the glutamate-NO-cGMP pathway and increases cGMP formation (Figure 1). The NMDA-induced increase in extracellular cGMP in cerebellum is significantly lower in hyperammonemic rats than in control rats[42], indicating that chronic hyperammonemia impairs the function of the glutamate-NO-cGMP pathway in rat cerebellum *in vivo*.

To assess whether the impairment occurs at the level of activation of soluble guanylate cyclase by NO, a NO-generating agent, SNAP (Figure 1) was administered through the microdialysis probe to directly activate guanylate cyclase. The increase in extracellular cGMP induced by SNAP was also significantly reduced in hyperammonemic rats. This indicates that chronic moderate hyperammonemia impairs activation of soluble guanylate cyclase by NO and function of the glutamate-NO-cGMP pathway in cerebellum *in vivo*.

Chronic liver failure induced by portacaval anastomosis, also impairs the glutamate-NO-cGMP pathway in cerebellum *in vivo*, as shown by brain microdialysis in freely moving rats by Monfort *et al*[43]. NMDA-induced increase in extracellular cGMP in cerebellum was significantly lower in rats with portacaval anastomosis than in control rats. These results indicate that the function of the glutamate-NO-cGMP pathway is impaired in cerebellum *in vivo* in animal models of HE. Moreover, one of the steps of the pathway affected is the activation of soluble guanylate cyclase by NO.

To assess whether activation of soluble guanylate cyclase by NO is also altered in cerebellum of cirrhotic patients with HE, we measured the activation of soluble guanylate cyclase by the NO-generating agent SNAP in homogenates of cerebellum from controls and cirrhotic patients who died of hepatic coma. The activation of guanylate cyclase by the NO-generating agent SNAP was significantly lower in cerebellum from cirrhotic patients than in cerebellum from controls[44].

The above results show that animal models of HE reproduce faithfully the alterations in the modulation of guanylate cyclase by NO present in cerebellum of patients died of HE, which supports the idea that hyperammonemia is responsible for these alterations.

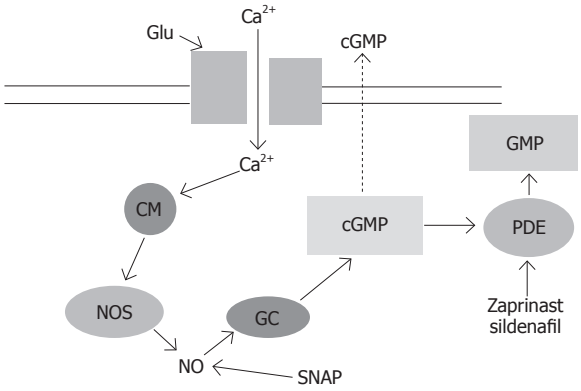

Figure 1 Glutamate-nitric oxide-cyclic GMP pathway. Activation of ionotropic (mainly NMDA) glutamate receptors leads to increased intracellular calcium (Ca^{2+}) which after binding to calmodulin (CM), activates nitric oxide synthase (NOS), leading to increased production of nitric oxide (NO), which in turn activates soluble guanylate cyclase (sGC), resulting in increased formation of cGMP. Part of the cGMP formed is released to the extracellular space. Soluble guanylate cyclase may be also activated by agents that generate NO such as SNAP. cGMP is degraded by phosphodiesterase (PDE) that may be inhibited by zaprinast or sildenafil.

LEARNING ABILITY IS IMPAIRED IN ANIMAL MODELS OF HE AND RESTORED BY PHARMACOLOGICAL NORMALIZATION OF THE FUNCTION OF THE GLUTAMATE-NITRIC OXIDE-CGMP PATHWAY AND CGMP IN BRAIN

Cognitive function and learning ability are impaired in patients with liver disease (see above) and in animal models of HE[35-37]. The glutamate-nitric oxide-cGMP pathway and cGMP modulate some forms of learning and memory. It is therefore likely that the impairment in the function of the glutamate-nitric oxide-cGMP pathway in brain may contribute to the cognitive impairment in HE patients.

We hypothesize that the alterations in the function of the glutamate-nitric oxide-cGMP pathway and the decrease in extracellular cGMP in brain may be responsible for the impairment in learning ability and intellectual function in HE patients, and that pharmacological modulation of extracellular cGMP concentration may restore learning ability in patients with hyperammonemia and HE.

To assess this possibility we tested whether pharmacological treatments directed to increase extracellular cGMP in brain are able to reverse the impairment in learning ability in rat models of HE. We were able to increase extracellular cGMP and completely restore learning ability of the rats by using three different treatments: continuous intracerebral administration of zaprinast, an inhibitor of the phosphodiesterase that degrades cGMP[36]; chronic oral administration of sildenafil, an inhibitor of the phosphodiesterase that crosses the blood-brain barrier[35], and continuous intracerebral administration of cGMP[36].

The above data indicate that the impairment of learning ability (at least of the ability to learn the Y maze conditional discrimination task) in animal models of HE is due to impairment of the glutamate-nitric oxide-cGMP

pathway. As the function of this pathway is also altered in brain of patients with liver cirrhosis, this alteration should also contribute to the cognitive impairment in these patients.

Increasing extracellular cGMP by pharmacological means may be a new therapeutic approach to improve learning and memory performance both in patients with evident HE and cognitive impairment and in patients with minimal HE who present reduced performance in psychometric tests.

HYPOLOCOMOTION IN ANIMAL MODELS OF HE IS DUE TO ALTERED ACTIVATION OF METABOTROPIC GLUTAMATE RECEPTORS IN THE CEREBRAL SUBSTANTIA NIGRA PARS RETICULATA

The neurological alterations in patients with HE also include alterations in motor function and coordination. One of the early alterations in patients with liver disease is psychomotor slowing. Jover *et al*[29] reported that 41% of patients with liver cirrhosis show hypokinesia, which is attributed to alterations in basal ganglia. These patients may also present bradykinesia. Rats with chronic liver failure due to portacaval anastomosis also show reduced motor activity which is similar to the motor slowing, hypokinesia and bradykinesia present in patients with HE[38,45-48]. We used this animal model to study the mechanisms involved in motor alterations in HE patients.

The neuronal circuits between basal ganglia and prefrontal cortex are essential in the modulation of motor function. This network includes basal ganglia, motor thalamus and cerebral cortex (Figure 2). The basal ganglia (including nucleus accumbens) produces signals that go to the thalamus which sends signals to the cortex to modulate movement execution. The signals originated in the thalamus are modulated by substantia nigra pars reticulata (SNr), which sends inhibitory projections to the ventromedial thalamus (VMT).

Patients with liver disease show hyperintensities in magnetic resonance imaging in basal ganglia nuclei[49-51], suggesting altered function of these nuclei, which is also supported by PET studies[52]. It has been suggested that the motor symptoms of HE are a consequence of basal ganglia dysfunction and alterations affecting the function of the neuronal circuits between basal ganglia and cerebral cortex[5,49-53].

The motor activity mediated by the basal ganglia-thalamus-cortex circuit is modulated by glutamatergic neurotransmission in nucleus accumbens and SNr (Figure 2). Activating metabotropic glutamate receptors in nucleus accumbens induces motor activity. Activation of glutamate receptors in SNr induces hypolocomotion in normal rats[54-56], while glutamate receptor antagonists administered in SNr induce hyperlocomotion[56,57]. Alterations in glutamatergic neurotransmission in SNr therefore may contribute to the psychomotor slowing and hypokinesia in HE patients.

We analysed the neurochemical alterations in the basal ganglia-thalamus-cortex circuit by *in vivo* brain microdialysis in rats with portacaval anastomosis and correlated the alterations in neurotransmitters (glutamate, GABA) with the alterations in motor function. Moreover we tried to normalize motor function of rats with portacaval anastomosis by pharmacologically modulating glutamatergic neurotransmission.

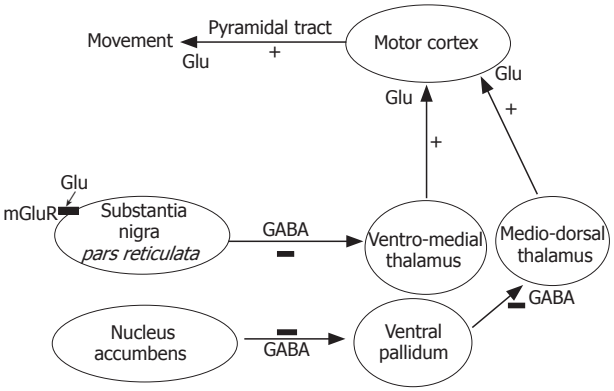

Figure 2 Hypolocomotion in animal models of HE is due to increased extracellular glutamate and activation of metabotropic glutamate receptors in substantia nigra pars reticulata. Activation of metabotropic glutamate receptors (mGluRs) in nucleus accumbens induces motor activity by activating a neuronal circuit involving ventral pallidum, medio-dorsal thalamus and motor cortex. Activation of mGluRs in substantia nigra pars reticulata (SNr) induces hypolocomotion by activating a neuronal circuit involving ventro-medial thalamus and motor cortex. In rats with HE due to chronic liver failure (portacaval anastomosis), the extracellular concentration of glutamate is significantly increased (15-fold) in SNr, resulting in increased activation of mGluR1 which is responsible for hypolocomotion in these rats. Blocking mGluR1 in SNr with specific antagonists increases motor activity in rats with chronic liver failure up to levels similar to those in normal rats.

Extracellular glutamate is significantly increased (15-fold) in SNr of rats with portacaval shunt (PCS) and its motor activity is reduced by 40%. There is a significant negative correlation between locomotor activity and extracellular glutamate in SNr, indicating that increased glutamate is responsible for hypolocomotion in PCS rats[38].

We hypothesize that hypolocomotion in PCS rats is due to over-activation of metabotropic glutamate receptors (mGluRs) by the increased extracellular glutamate. To assess this possibility, we tested whether blocking mGluR1 with the antagonist CPCCOEt can normalize the motor activity in PCS rats and found that blocking mGluR1 does not affect the motor activity in control rats but increases it in PCS rats to reach the same motor activity than control rats[38]. The above data show that increased activation of the metabotropic glutamate receptor mGluR1 in SNr is responsible for hypolocomotion in rats with chronic liver failure. We studied the possible mechanisms involved in this increased activation. The increased activation of mGluR1 is not due to increased amount of the receptor, which is significantly reduced in SNr of PCS rats[38], indicating that enhanced activation of the metabotropic receptor is due to the increase in the extracellular concentration of glutamate.

The increase in extracellular glutamate could be due to increased release, reduced uptake or both. We analysed the content of the two main transporters that take up glutamate: EAAC-1 and GLT-1. The content of both

transporters is reduced by 23%-27%[38]. The reduced content of glutamate transporters may contribute to increased extracellular glutamate in SNr. Enhanced glutamate release could also contribute to the increased extracellular glutamate.

The increased activation of metabotropic glutamate receptors in SNr results in altered function of the whole basal ganglia-thalamus-cerebral cortex circuit (Figure 2), and therefore in hypolocomotion. Increased mGluR1 activation in SNr leads to increased GABA concentration in ventro-medial thalamus, which mediates hypolocomotion in PCS rats. Blocking mGluR1 in SNr normalizes GABA in ventro-medial thalamus and locomotion. The circuit by which SNr modulates motor activity also involves glutamate in motor cortex. Blockade of mGluR1 in SNr increases glutamate in motor cortex and activity.

There are reports supporting that changes similar to those summarized above in rats with chronic liver failure also may occur in patients with chronic liver disease. Alterations in the function of basal ganglia in liver cirrhosis are supported by the hyperintensities in magnetic resonance images found consistently in these patients[49-51]. Altered function of the thalamus is also supported by PET studies in patients with HE[52]. It is therefore likely that excessive activation of metabotropic glutamate receptors in SNr may be also involved in the psychomotor slowing and hypokinesia in patients with HE.

These studies may have clinical implications. Blocking mGluR1 in SNr normalizes motor activity in a rat model of HE, suggesting that a similar treatment for patients with HE can be used to treat psychomotor slowing and hypokinesia in these patients. However, the possible effects of mGluR1 antagonists on other cerebral functions should be previously evaluated. Each type of glutamate receptors and their associated signal transduction pathways may be expressed in different brain areas where they may modulate different cerebral functions. This implies that, for example, blocking mGluR1 in SNr may restore motor function in patients with liver disease, but blocking it in another brain area may impair some other cerebral functions. Before trying to apply these treatments in humans, careful studies in animal models are therefore required to analyse the possible secondary effects, and ideally, if possible, to develop appropriate delivery procedures allowing to modulate specific receptors or pathways in specific brain areas without affecting its function in other areas.

In summary, the studies reviewed here show that alterations in signal transduction pathways associated to the NMDA type of ionotropic glutamate receptors are involved in cognitive impairment in HE while alterations in activation of metabotropic glutamate receptors are involved in the motor alterations. Moreover, pharmacological manipulation of the altered pathways and receptors can normalize both cognitive and motor functions in animal models of HE. These studies are setting the basis for designing therapeutic procedures to specifically treat individual neurological alterations in patients with HE.

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S- Editor Wang J **L- Editor** Wang XL **E- Editor** Bai SH

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World J Gastroenterol 2006 December 28; 12(48): 7744-7752
World Journal of Gastroenterology ISSN 1007-9327

REVIEW

Food allergy in gastroenterologic diseases: Review of literature

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Received: 2006-09-11 Accepted: 2006-11-14

Abstract

Food allergy is a common and increasing problem worldwide. The newly-found knowledge might provide novel experimental strategies, especially for laboratory diagnosis. Approximately 20% of the population alters their diet for a perceived adverse reaction to food, but the application of double-blind placebo-controlled oral food challenge, the "gold standard" for diagnosis of food allergy, shows that questionnaire-based studies overestimate the prevalence of food allergies. The clinical disorders determined by adverse reactions to food can be classified on the basis of immunologic or nonimmunologic mechanisms and the organ system or systems affected. Diagnosis of food allergy is based on clinical history, skin prick tests, and laboratory tests to detect serum-food specific IgE, elimination diets and challenges. The primary therapy for food allergy is to avoid the responsible food. Antihistamines might partially relieve oral allergy syndrome and IgE-mediated skin symptoms, but they do not block systemic reactions. Systemic corticosteroids are generally effective in treating chronic IgE-mediated disorders. Epinephrine is the mainstay of treatment for anaphylaxis. Experimental therapies for IgE-mediated food allergy have been evaluated, such as humanized IgG anti-IgE antibodies and allergen specific immunotherapy.

Key words: Food intolerance; Food allergy; Skin prick test; Serum food-specific IgE; Oral food challenges

Mansueto P, Montalto G, Pacor ML, Esposito-Pellitteri M, Ditta V, Lo Bianco C, Leto-Barone SM, Di Lorenzo G. Food allergy in gastroenterologic diseases: Review of literature. *World J Gastroenterol* 2006; 12(48): 7744-7752

http://www.wjgnet.com/1007-9327/12/7744.asp

INTRODUCTION

Food allergy is recognized as a common worldwide problem, and, like other atopic disorders, its incidence seems to increase. Moreover, food-related allergic disorders are the leading cause of anaphylactic reactions treated in the emergency departments in a number of countries, accounting for approximately 30 000 emergency department visits, and 150-200 deaths each year, and the public opinion has become increasingly aware of the problem[1,2]. In the past years, investigations of allergic food proteins and related immunological responses have moved to the molecular level, and the newly-found knowledge might provide novel experimental strategies for the laboratory diagnosis and the immuno-modulatory control of food-induced allergic reactions[1,3-5].

Recently, the European Academy of Allergy and Clinical Immunology task force published a revised nomenclature for allergy. Adverse food reactions, defined as "food hypersensitivities", include any abnormal reaction resulting from the ingestion of a food, and it might be the result of food intolerance, defined as "non-allergic food hypersensitivities", excluding immunologic mechanisms, or food allergy, defined as "allergic food hypersensitivities", including clear, or strongly suspected, immunologic mechanisms[6]. Our review focused on the food allergy.

EPIDEMIOLOGY AND PATHOGENESIS

Epidemiology

Approximately 20% of the population alters their diet for a perceived adverse reaction to food, but the application of double-blind placebo-controlled oral food challenge, considered as the gold standard for diagnosis of food allergy, shows that questionnaire-based studies overestimate the prevalence of food allergies and food intolerance[7-9].

In the United States, approximately 6% of infants and young children and 3.7% of adults have food allergy. In young children, the most common causal foods are cow's milk, egg, peanut, wheat, soy, tree nuts, fish, and shellfish. In adults, the most common causal foods are shellfish, peanut, tree nuts and fish[7,10-12]. Early childhood allergy to milk, egg, soy, and wheat are usually resolved by school age (approximately 80%), whereas peanut, tree nuts and

seafood allergies are generally considered permanent. In Europe, early childhood allergy to cow's milk has an incidence of approximately 2%. The relatively high prevalence of peanut allergy in British children (0.5%) ("Americanised" eating habit) is not reflected in the results from other European countries. Cow's milk, egg and orange seem to be the most common causes of allergy in European infants and children. As the children become adults, allergy to milk and eggs become less frequent. In adults, the allergies appear toward pollen-related food, i.e. *Compositae*-celery, birch-apple and birch-peaches. Adult European population presents a prevalence of food allergy/intolerance of approximately 5%[7,13].

Unfortunately, data from many parts of Asia are still lacking. However, the prevalence of food allergy in Asia seems to be low, but is likely to increase with the global increase of allergy. Asia is unique because of the many different cultures and eating habits, with the existence of unique food allergens. Peanut and tree nuts are rarely the cause of allergic reactions in this area[14]. In a population-based study carried out in the United Kingdom establishing reported food problems and sensitization among 11- and 15-year-old children, the prevalence of food hypersensitivity was 1.4% and 2.1% for the 11- and 15-year-olds, respectively[15] on the basis of a combination of a clear history of previous reactions, a positive skin prick test response, a positive open food challenge result, and a positive double-blind placebo-controlled food challenge.

Pathogenesis

Food allergy represents an abnormal response of the mucosal immune system to antigens delivered through the oral route[6]. The healthy gastrointestinal mucosal immune system encounters enormous quantities of antigen on a daily basis and generally suppresses immune reactivity to harmless foreign antigens (food proteins and commensal bacteria), although it is fully capable of mounting a brisk protective response against dangerous pathogens. The process by which the gastrointestinal immune system avoids attacking harmless antigens is termed "oral tolerance"[16,17]. Food allergy might result from a failure in oral tolerance to food while they are being ingested (class 1 food allergy) or from a sensitization to allergens recognized instead during respiratory exposure (class 2 food allergy). Class 1 food allergy is typically related to food proteins, generally stable to digestion, which is encountered by infants or children at a presumed immunological immaturity. In contrast, class 2 food allergy is the result of a sensitization to protein susceptible to enzymatic degradation, encountered in the respiratory tract, such as pollens, resulting in an IgE antibody production that recognize homologous epitopes on food proteins of plant origin (i.e. pollen-food related syndrome)[10,11,18,19].

Gut barrier: The gastrointestinal mucosal barrier is a complex of physical (mucus, acid, enzymes, bile salts, and epithelial cell tight junctions) and immunologic structures-both "innate" (natural killer cells, polymorphonuclear leukocytes, macrophages, epithelial cells, and toll-like receptors), and "adaptive" (intraepithelial and *lamina propria* lymphocytes, Peyer's patches, secretory immunoglobulin type A [sIgA], and cytokines)-which all serve to destroy antigens and to render antigens nonimmunogenic[16,20-22]. Alteration of the gut barrier might lead to food allergy. Developmental immaturity of components of the gut barrier (enzymatic activity and sIgA) might account for the increased prevalence of food allergy in infancy[23]. Despite the evolution of this barrier, about 2% of ingested food antigens, both particulate and soluble, are adsorbed by the follicle associated epithelium (M cells), overlying Peyer's patch and the intestinal epithelial cells, respectively, and transported throughout the normal mature gut, but they infrequently induce clinical symptoms, because tolerance develops in most individuals[24,25].

Oral tolerance induction: The immunologic mechanisms involved in oral tolerance induction have not been fully elucidated. Antigen-presenting cells, epithelial and dendritic cells, and regulatory T cells play a central role. Intestinal epithelial cells, as non-professional antigen-presenting cells, process luminal soluble dietary antigen and present it, on an MHC class Ⅱ complex, selectively to $CD8^+$ suppressor T cells, thus playing a role in local control and suppression of immune responses. Dendritic cells, residing within the *lamina propria*, are professional antigen presenting cells that secrete IL-4, a pro-inflammatory T_H2 cytokine, and IL-10, an anti-inflammatory cytokine. However, the specific role of these cells in directing the balance between active immunity and food tolerance in the intestine, depends on the cytokine microenvironment and the expression of costimulatory molecules. Five regulatory T cells have been identified in association with intestinal immunity: T_H3 cells, a population of $CD4^+$ cells that secrete transforming growth factor β (TGF-β), might play an important role in oral tolerance, inducing T cell suppression and promoting B-cell switching to sIgA production; T_R1 cells, $CD4^+$ cells that secrete IL-10 and suppresses the antigen-specific immune responses; $CD4^+CD25^+$ regulatory T cells, together with $CD8^+$ suppressor T cells, that are both capable of suppressing the effector T cells; and γδ T cells, whose role in oral food tolerance is still unclear[26,27]. Dose of dietary antigens, frequency of exposure, and chemophysical properties of food proteins, might also influence the tolerance induction. Particularly in mice, oral food tolerance has been induced after administration of either a single dose or repeated lower doses of antigen. These two forms of tolerance, termed high-dose and low-dose tolerance, respectively, might be mediated by different mechanisms: by the activation of regulatory T cells (T_H3, T_R1, and $CD4^+CD25^+$), with suppressor function, and by the anergy or delection of effector T cells. Anergy can occur through T-cell receptor activation in the absence of costimulatory signals provided by soluble cytokines or by interactions between costimulatory receptors on T cells and counter-receptors on antigen presenting cells. Delection occurs by means of FAS-mediated apoptosis of lymphocytes[28].

Commensal gut flora might also influence the mucosal immune response. Gut flora is largely established in the first 24 h. after birth and is dependent on maternal flora and local environment. Gut flora might enhance a T_H1 cytokine response, with secretion of interferon-γ (IFN-γ) that inhibits T_H2 responses. However, in adults, commensal gut flora seems to be less important in the regulation of mucosal immune response[29,30].

Table 1 Pathophysiologic classification of adverse reactions to food

Non allergic food hypersensitivities
Toxic
Pharmacologic
Metabolic disorders
Idiosyncratic responses
Allergic food hypersensitivities
Allergy
IgE-mediated
Non IgE-associated
Mixed IgE-mediated/non IgE-mediated

Table 2 Clinical classification of allergic disorders induced by food

Gastrointestinal food-induced allergic disorders
Pollen-food allergy syndrome
Allergic eosinophilic esophagitis
Allergic eosinophilic gastroenteritis
Food protein-induced enterocolitis, proctocolitis, and enteropathy
Celiac disease
Infantile colic
Gastrointestinal anaphylaxis
Cutaneous food-induced allergic disorders
Acute urticaria and angioedema
Atopic dermatitis
Dermatitis herpetiformis
Respiratory food-induced allergic disorders
Rhinoconjunctivitis
Bronchial asthma
Systemic food-induced allergic disorders
Generalized anaphylaxis
Food-associated exercise-induced anaphylaxis

Food allergens: The regional dietary habits and methods of food preparation clearly play a role in the prevalence of specific food allergies in various countries around the world[31].

The major food allergens identified as class 1 allergens are water-soluble glycoproteins, 10 to 60 kilo-Dalton in size, that are stable to heat, acid, and proteases. Cooking can reduce the allergenicity of certain food proteins; conversely, heating can increase the allergenicity of other food proteins, through the induction of covalent modifications that lead to new antigens or improved stability[11,32]. The class 2 food allergens are presumably comprised of conformational epitopes and therefore are highly heat-labile, susceptible to enzymatic degradation and difficult to isolate. An example of a class 2 allergens is the birch pollen Bet v 1, that can induce sensitization through the respiratory tract and results in oral symptoms of pruritus to homologous class 2 allergens in raw apple (Mal d 1) or carrot (Dau c 1)[19,33].

Genetic of the host: Studies examining potential associations of specific HLA antigens with allergies to different food show variable results. No difference was observed when HLA-A, HLA-B, and HLA-C locus antigen were compared between patients with food allergy and control subjects. However, when individuals with peanut allergy and unrelated control subjects were typed for the HLA-class Ⅱ genotypes, DRB1*08, DRB1*08/12tyr16, and DQB1*04 were found at higher frequency in those with peanut allergy than in control subjects. These findings indicate that allergic reactions to peanut are in part under genetic control. Additional genes might be involved in the overall expression of food allergy and are under investigations[34].

CLINICAL CLASSIFICATION

The clinical disorders determined by adverse reactions to food (or food hypersensitivity) can be classified on the basis of nonimmunologic or immunologic causes (Table 1), and the organ system or systems affected (Table 2)[6]. Food intolerance by toxic and pharmacologic reactions is due to toxic contaminants (histamine in scombroid fish poisoning, and bacterial food poisoning) or pharmacological substances within the food (tyramine in aged cheeses) which can affect most healthy individuals when given at appropriate doses. Food intolerance may also be attributed to some unique physiologic characteristics of the host, such as a metabolic disorder (lactase deficiency), or an idiosyncratic response. Instead, food allergy is defined as an adverse reaction to food that is immunologically mediated, and involves specific IgE or non-IgE (T cell-mediated) mechanisms or both[6].

Gastrointestinal food-induced allergic disorders

Various gastrointestinal food-induced allergic disorders share the same symptoms, such as vomiting, abdominal distension and pain, and diarrhoea, but they can be differentiated by patterns of illness and diagnostic tests.

Pollen-food allergy syndrome: Pollen-food allergy syndrome (or oral allergy syndrome): is an IgE-mediated food adverse reaction, elicited by a variety of plant-derived food proteins, especially concentrated in the peel, which cross-react with airborne allergens, including birch, ragweed, and mugwort pollens. It is characterized by mild pruritus, tingling, and/or angioedema of the lips, tongue, palate or oropharynx, occasional sensation of tightness in the throat, and rarely systemic symptoms, because the allergens responsible for these reactions are easily broken down by heat or gastric enzymes, and thus are not absorbed by the gastrointestinal mucosa. Reactions to all related food are rare, but sensitivity to more than one is common. Diagnosis is based on clinical history, positive skin prick test responses to fresh food and relevant airborne proteins, and, if necessary, on an oral challenge, positive with fresh food and negative with cooked food (see above)[35-37].

Allergic eosinophilic esophagitis: Allergic eosinophilic esophagitis is an IgE- or non-IgE-mediated, or both, food adverse reactions, seen most frequently during infancy through adolescence, characterized by gastroesophageal reflux, excessive spitting-up or emesis, dysphagia, intermittent abdominal pain, failure to respond to conventional reflux medications, and peripheral blood eosinophilia. Diagnosis is based on clinical history, skin prick tests, endoscopy with biopsy,

elimination diet and challenge. Patients who are not appropriately treated might develop fibrosis, with subsequent esophageal stricture, and Barrett's esophagitis[38,39].

Allergic eosinophilic gastroenteritis: Allergic eosinophilic gastroenteritis is an IgE- or non-IgE-mediated, or both, food adverse reactions, being diagnosed more frequently in adults, characterized by early satiety, intermittent vomiting, recurrent abdominal pain, blood loss in the stools, iron-deficiency anemia, and protein-losing enteropathy, with a peripheral blood eosinophilia. Clinical history, skin prick tests, endoscopy with biopsy, and elimination diet and challenge, are required[40,41] for the diagnosis.

Food protein-induced enterocolitis, proctocolitis, and enteropathy: Food protein-induced enterocolitis, proctocolitis, and enteropathy is a non-IgE-mediated (T cell-mediated) disorders, most commonly seen in infants before 3 mo of age, provoked by food proteins in maternal breast or cow's milk or soy protein-based formulas, characterized by nausea, protracted projectile vomiting, that begins about 1-3 h. after allergen ingestion, abdominal distension, flatulence, diarrhoea (steatorrhoea), sometimes with dehydratation, acidemia, methemoglobinemia, weight loss and gross or occult blood in stool mixed with mucus. In these patients, skin prick test responses are negative. Endoscopy and biopsy are often required. In patients affected by food protein-induced enteropathy, biopsy reveals a patchy villous atrophy, a prominent mononuclear round cell infiltrate, and few eosinophils. Elimination of food proteins leads to the clearing of symptoms in 24-72 h. Challenge induces recurrent vomiting or bleeding within 72 h.[42-46].

Celiac disease: Celiac disease (or gluten-sensitive enteropathy) is a more extensive enteropathy leading to malabsorption, associated with sensitivity to gliadin, found in wheat, rye and barley. Diagnosis is based on celiac IgA, anti-gliadin and anti-transglutaminase antibodies detection, endoscopy and biopsy, elimination diet, with resolution of symptoms and food challenge, if necessary[47,48].

Infantile colic: Infantile colic is due to food hypersensitivity in a minority of infants presenting with this disorder characterized by paroxysmal fussiness with inconsolable agonized crying, it generally develops in the first 2-4 wk of life, and persists through the third to fourth month of life. Diagnosis is based on the implementation of several brief trials of hypoallergenic formula[49,50].

Gastrointestinal anaphylaxis: Gastrointestinal anaphylaxis is an IgE-mediated reaction, food associated, not exercise-induced, characterized by rapid onset of nausea, vomiting, cramps, abdominal pain, and diarrhoea, often involving other target organs such as skin and respiratory tract. Diagnosis is established according to the clinical history, positive skin prick test or radioallergosorbent test (RAST) responses, and if necessary, based on an oral challenge[11,51].

Cutaneous and respiratory food-induced allergic disorders

IgE-, non-IgE- and mixed IgE- and non-IgE-mediated adverse reactions to food can induce a variety of cutaneous disorders. The most common cutaneous disorder of food-induced allergic reactions is "acute" urticaria and angioedema (symptoms lasting < 6 wk), whereas, food allergy causes infrequently "chronic" urticaria and angioedema (symptoms lasting > 6 wk)[52,53].

Atopic dermatitis: Atopic dermatitis is a form of eczema that generally begins in early infancy, characterized by typical distribution, extreme pruritus, and a chronically relapsing course. In about 35% of children with moderate-to-severe disease, food allergens specific serum IgE antibodies against cow's milk, egg, soya and wheat are demonstrable, and the ingestion of specific food might evoke a marked worsening of cutaneous lesions[54,55].

Dermatitis herpetiformis: Dermatitis herpetiformis is a rare chronic skin disorder, associated with gluten-sensitive enteropathy, characterized by a chronic, intensely pruritic, papulovesicular rash, symmetrically distributed over the extensor surfaces and buttocks. It can be clearly distinguished from the other subepidermal blistering eruptions by gastrointestinal, immunologic and histologic criteria. Both enteropathy and the dermatologic findings disappear with a gluten-free diet, therefore, dermatitis herpetiformis is thought to be the specific dermatologic finding of celiac disease[56].

Food allergy can also induce a number of disorders in the respiratory tract. Acute respiratory symptoms, caused by food allergy generally represent isolated IgE-mediated reactions, whereas chronic respiratory symptoms represent a mix of IgE- and non-IgE-mediated reactions. Isolated rhinoconjunctivitis and bronchial asthma are rarely the result of food-induced allergic reactions, although they frequently occur in association with other food allergy symptoms. However, food allergy was found to be a major risk factor for severe life-threatening asthma. Food-induced asthmatic symptoms should be suspected in patients with refractory asthma and a history of atopic dermatitis, gastroesophageal reflux, food allergy or a history of positive skin prick test responses to a kind of food[10,57-59].

Systemic food-induced allergic disorders

IgE-, non-IgE- and mixed IgE- and non-IgE-mediated adverse reactions to food can also induce systemic disorders.

Generalized anaphylaxis: Generalized anaphylaxis is an IgE-mediated food adverse reaction, accounting for at least one third to one half of anaphylaxis cases seen in hospital emergency departments. In addition to variable expression of cutaneous (itching, flushing and urticaria), respiratory (asthma) and gastrointestinal (nausea, vomiting, abdominal pain, and diarrhea) symptoms, patients might have cardiovascular symptoms, such as hypotension, cyanosis, vascular collapse and cardiac dysrhythmias. Most of fatal food-induced anaphylaxis were adolescents or young adults, with previous histories of reacting to the implicated food (usually not life-threatening), and all were affected by underlying asthma. Peanuts, tree nuts and seafood were responsible for the vast majority of the fatalities in the United States. Aspirin, exercise and alcohol can increase the risk[60-62].

Food-associated and exercise-induced anaphylaxis: Food-associated and exercise-induced anaphylaxis is a form of anaphylaxis that occurs only when the patient (generally women aged 15-30 years) exercises within 2-4 h. of ingesting food. Crustaceans and wheat are the two commonest but other food can be implicated. In the absence of exercise, the patient can ingest the food without any apparent reaction. It might account for up to one

half of the cases of exercise-induced anaphylaxis. Diagnosis is based on patient history and the demonstration of food-specific serum IgE antibodies. Food dependent and exercise-induced anaphylaxis should be considered in young children with exercise-induced anaphylaxis of unknown origin[63,64].

DIAGNOSIS

The evaluation of a patient with a possible allergic food reaction begins through clinical history and a complete physical examination to consider a potentially broad differential diagnosis between food-induced allergic clinical disorders and other gastrointestinal disease, such as food intolerance (toxic and pharmacological effects or metabolic disorders), infections (viral, bacterial and parasitic), celiac disease, inflammatory bowel diseases, bowel ischaemia, gallbladder disease, pancreatic insufficiency, and gastrointestinal neoplasms[7,10,11]. The medical history continues to be a mainstay in the diagnostic process, and might determine the possible causal food, quantity ingested, time course of reaction, ancillary factors (aspirin, exercise and alcohol) and reaction characteristics. However, the identification of suspected food is difficult because food is ingested throughout the day and symptoms that arise soon after an ingestion might be wrongly attributed to food allergy, or attributed to the wrong food. Diet records and symptom diaries can be a useful supplement to a medical history, especially in chronic disorders. From a diagnostic point of view, it is helpful to categorize food hypersensitivity disorders by the mechanism of response and the predominant target organ. IgE-mediated reactions are typically rapid in onset, whereas non-IgE-mediated disorders become evident after allergen ingestion. Some disorders might involve both IgE- and non-IgE-mediated mechanisms and vary in their time of onset. In other words, acute symptoms, such as acute urticaria after ingestion of a food, are likely caused by food allergy, whereas chronic symptoms (chronic urticaria and asthma) are less likely attributable solely to food allergy. Certain disorders are commonly associated with food allergy, such as moderate-to-severe atopic dermatitis. For other disorders such as chronic urticaria, suspicions about particular food are notoriously inaccurate, and are only verified in about 30% of cases. In some cases, confirmation of a diagnosis of allergic food reaction requires invasive tests, such as endoscopy, but usually the diagnosis relies on food-specific IgE determination (or confirmations of its absence), results of elimination diets, and responses to oral food challenges[7,10,11].

Skin prick test

For IgE-mediated disorders, skin prick tests provide a rapid mean to detect sensitization. This almost painless procedure allows the tested protein to interact with food-specific IgE on the surface of skin mast cells. If the antibody is present, mast cells degranulate and release mediators that rapidly cause localised vasodilation, angioedema and wheal and flare. While the patient discontinued antihistamines for an appropriate length of time, a device, such as a lancet, plastic probe or tip of a small gauge needle, is pressed through a commercial extract of food and a positive (histamine) and negative (saline-glycerine) controls into the epidermis. Allergens eliciting within 15 min a wheal at least 3 mm larger than that produced by the negative control are considered positive, indicating the possibility that the patients have symptomatic reactivity to the specific food, with strongly positive results implying a greater likelihood of clinical reactivity. On the other hand, negative skin prick test responses essentially confirm the absence of IgE-mediated allergic reactivity. To maximize the utility of skin prick test results, clinical history and disease pathophysiology are required. For example, a positive skin prick test response may be considered confirmatory for the diagnosis when combined with a recent and clear-cut history of a food-induced allergic reaction to the tested food[65-69].

When evaluating allergy to fruits and vegetables, commercially prepared extracts are often inadequate because they are prone to degradation, and therefore the fresh food might be used for prick-by-prick test[70]. A number of investigators have examined the use of the "atopy patch test" in addition to skin prick test for the diagnosis of non-IgE-mediated food allergy, with delayed reactions to food, but at this time, there are no standardized reagents or methods of application and interpretation. Thus, its diagnostic accuracy remains still controversial, especially in older children[71].

Detection of serum food-specific IgE

Laboratory tests to determine serum food-specific IgE antibodies (RAST or, more recently, the CAP System FEIA, or UniCAP [Pharmacia and Upjohn Diagnostics, Uppsala, Sweden], and others) provide another modality to evaluate IgE-mediated food allergy. Manufactures and substrates vary, and results can be classified into class one to six, or arbitrary units of concentration (kU$_A$/L). Increasingly higher concentrations of food-specific IgE correlate with an increasing likelihood of clinical reaction[72-74]. No conclusive studies indicate that determination of specific IgE-binding epitopes on an allergen might provide increased diagnostic utility. Further analysis revealed that determining epitope-specific binding might correlate with clinical reactivity better than quantitative IgE values to the whole protein. Moreover, evaluating the number of allergenic epitopes bound by the IgE antibodies might be useful for predicting the clinical severity of food-induced allergic reaction[75,76].

Other laboratory tests

When evaluating patients with gastrointestinal symptoms, suspecting a food hypersensitivities, a number of other standard laboratory studies might be useful. Patients with allergic eosinophilic esophagitis and allergic eosinophilic gastroenteritis have peripheral eosinophilia, and patients with severe allergic eosinophilic gastroenteritis might have anemia, blood in the stool, and decreased serum protein, albumin and IgG levels (with preservation of IgM and IgA)[77-79]. Endoscopy and biopsy are the most definitive approaches for diagnosing many of the gastrointestinal food hypersensitivities and might help the differential diagnoses. Greater than 10-20 eosinophils per 40 × high-power field in the esophagus is diagnostic of allergic eosi-

nophilic esophagitis, especially if the pH probe is normal and there is lack of responses to antireflux medication. Eosinophils are normally present in the gastric and intestinal mucosa, and therefore eosinophil number must be greater to make the diagnosis of allergic eosinophilic gastroenteritis. In these cases, diagnosis requires elimination of alternative diagnostic possibilities (parasites, inflammatory bowel disease)[77-79]. No conclusive studies suggest the possible usefulness of analyzing intestinal permeability by determining the 5-h. urinary excretion of [51Cr] EDTA, and inflammation markers, including histamine, eosinophilic cationic protein, tryptase, and calprotectin in gut lavage fluid[80].

Oral food challenge

Skin prick tests with food allergens and determination of serum food-specific IgE can detect "sensitization" (that is the presence of food-specific IgE), but because sensitization can exist without allergic clinical reactions (esophagitis, gastroenteritis, rhinitis and asthma), these tests generally cannot be used alone to diagnose food allergy. In this setting, it is important to consider also the clinical history and the results of oral food challenges. Skin prick tests and RAST are most valuable when they are negative, since their high sensitivity makes them about 95% accurate for discounting IgE-mediated reactions. The double-blind, placebo-controlled oral food challenge (DBPCFC) with gradually increasing amounts of the suspected food under observation over hours or days, is considered the "gold standard" test for the diagnosis of food allergy. The clinical history results, skin prick tests (RASTs) or both, indicate which food should be evaluated by DBPCFCs. Patients with histories of life-threatening anaphylaxis should be challenged only when the history and laboratory tests cannot conclusively determine the causative food. To increase the likelihood of a nonequivocal food challenge result, suspected food should be eliminated for 7-14 d before challenge and longer in some non-IgE-mediated gastrointestinal disorders (non-IgE mediated allergic eosinophilic esophagitis and allergic eosinophilic gastroenteritis). Medications that could interfere with the evaluation of food-induced symptoms (antihistamines and adrenergic bronchodilators) must be discontinued. The length of the observation period depends on the type of reaction suspected. Hypotension might occur in about 15% of these challenges, especially in patients affected by acute IgE-mediated reactions, enterocolitis syndrome, and severe atopic dermatitis, and therefore intravenous hydration therapy and supplies for resuscitation should be immediately available. The false negative rate of DBPCFC is about 3%, so negative challenges should always be followed by a supervised open or a single-blind oral food challenge[8,66,81].

MANAGEMENT

The primary therapy for food allergy is to avoid the causal food. In most countries, shortcomings on manufacturers and labelling, make it very difficult to identify allergens in commercial food products. Cross contamination, errors in packaged food shop, and restaurants are additional obstacles. Therefore, new food-labelling laws require simple terms to indicate the presence of major food allergens ("milk" instead of "casein"). Patients and care providers should be encouraged to obtain medical identification bracelets, taught to recognize symptoms, and instructed on using self-injectable epinephrine and activating emergency services. Clinical tolerance develops to most food allergens over time, except for peanuts, nuts and seafood. Periodic reintroduction of food allergens under physician supervision is warrant to determine whether clinical tolerance has develop[7,10-12].

There is a relationship between the decrease in serum food-specific IgE concentrations and the likelihood of developing tolerance. A greater decrease in serum food-specific IgE levels over a shorter period of time might be indicative of a greater likelihood of developing tolerance. The confirmation of this model and subsequent application in clinical practice would aid clinicians in the timing of food challenges and in providing prognostic information for patients and their families[82].

Medications

Various medications can provide relief for certain aspect of food-induced disorders[7,10-12].

Antihistamines might partially relieve symptoms of oral allergy syndrome and IgE-mediated skin symptoms, i.e. itching and rash, but do not block systemic reactions. Systemic corticosteroids are generally effective in treating chronic IgE-mediated disorders (atopic dermatitis). A course of corticosteroids can be used to reverse severe inflammatory symptoms, but the side effects of protracted use are unacceptable. Epinephrine is the mainstay of treatment for anaphylaxis. Intramuscolar injection allows more efficient absorption than the subcutaneous route[83].

Novel therapies for IgE-mediated food allergy have been evaluated. Subcutaneous injections of humanized IgG anti-IgE antibodies (TNX-901), that recognize and mask an epitope in the CH3 region of IgE responsible for the binding to the high affinity Fc epsilon receptor I (FCεRI) on basophils and mast cells, for the treatment of patients affected by peanut allergy, showed a long-term increase in the average amount of peanut tolerated, but 25% of subjects showed no improvement[84].

Another anti-IgE preparation (Omalizumab) has been approved for the treatment of persistent allergic asthma in patients who are poorly controlled with inhaled corticosteroids, but has not yet been evaluated for its efficacy in treating patients with peanut allergy. Theoretically, anti-IgE antibody therapy should be protective against multiple food allergens, although it would have to be administered indefinitely to maintain its protective effects[85].

No conclusive studies indicate that standard allergen specific immunotherapy for birch- or ragweed pollen-induced rhinitis might improve pollen-food allergy syndrome. The risk/benefit ratio of traditional immunotherapy for the treatment of peanut allergy was considered unacceptable, because the injection of food protein results in anaphylaxis. To address this problem, engineered proteins are altered to remove IgE-binging epitopes that trigger anaphylaxis, while T-cell epitopes that could induce toler-

ance to specific food allergen, are preserved[86,87]. Other immunotherapeutic strategies include use of engineered proteins lacking IgE-binding sites, immunomodulatory sequences being effective in reversing IgE-mediated sensitization, and engineered chimeric molecules forming complexes with allergen-specific IgE bound to mast cells and basophils, inhibiting their functions.

Some recent studies suggested that probiotics, commonly defined as live microorganisms (bacteria from the genera *Lactobacillus, Bifidobacterium*, *Escherichia*, *Enterococcus*, *Bacillus* and *Saccharomyces*), administered in adequate amounts, which confer a beneficial health effect on the host, might be useful in the treatment and prevention of food allergy. They might provide maturational signals for the gut-associated lymphoid tissue, balance the generation of pro- and anti-inflammatory cytokines, reduce the dietary antigen load by degrading and modifying macromolecules, reverse the increased intestinal permeability, characteristic of children with food allergy, normalization of the gut microecology, and enhance specific sIgA responses frequently defective in children with food allergy[5,88-92].

Prevention

Approaches to delay or prevent allergy through dietary manipulation have been considered. Some studies suggest a beneficial role for exclusive breast-feeding of infants at high risk for atopic diseases in the first 3-12 mo of life and avoidance of supplementation with cow's milk or soy formulas in favour of hypoallergenic formulas if breast-feeding is not possible[93].

Maternally ingested food can pass in immunologically intact form into breast milk and might induce reactions in infants. No conclusive studies indicate that manipulation of mother's diet during pregnancy or breast-feeding or the restriction of allergenic food from the infant's diet will prevent the development of food allergy[94]. The American Academy of Pediatrics recommends that high-risk infants (both parents and siblings atopic) be exclusively breast-fed, that lactating mothers avoid peanuts and nuts to avoid sensitization through breast milk, that the introduction of solid be delayed until 6 mo of age, and major allergens, such as peanuts, nuts and seafood, be introduced after 3 years of age[95].

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S- Editor Wang GP **L- Editor** Ma JY **E- Editor** Liu WF

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World J Gastroenterol 2006 December 28; 12(48): 7753-7757
World Journal of Gastroenterology ISSN 1007-9327

REVIEW

Mechanisms of regulation and function of G-protein-coupled receptor kinases

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Received: 2006-10-20 Accepted: 2006-11-23

Abstract

G-protein-coupled receptor kinases (GRKs) interact with the agonist-activated form of G-protein-coupled receptor (GPCR) to affect receptor phosphorylation and to initiate profound impairment of receptor signaling, or desensitization. GPCR forms the largest family of cell surface receptors, and defects in GRK function have the potential consequence to affect GPCR-stimulated biological responses in many pathological situations.

Key words: G-protein-coupled receptor kinases; G-protein-coupled receptor; Signal; Transduction; Phosphorylation

Yang W, Xia SH. Mechanisms of regulation and function of G-protein-coupled receptor kinases. *World J Gastroenterol* 2006; 12(48): 7753-7757

http://www.wjgnet.com/1007-9327/12/7753.asp

INTRODUCTION

G-protein-coupled receptor kinases (GRKs) are key modulators of G-protein-coupled receptor (GPCR) signaling. They constitute a family of seven mammalian serine-threonine protein kinases that phosphorylate agonist-bound receptor. GRKs-mediated receptor phosphorylation rapidly initiates profound impairment of receptor signaling and desensitization. Activity of GRKs and subcellular targeting is tightly regulated by interaction with receptor domains, G protein subunits, lipids, anchoring proteins and calcium-sensitive proteins. Moreover, GRK phosphorylation by several other kinases and autophosphorylation have recently been shown to modulate its functionality. This review summarizes our current knowledge of GRKs-regulatory mechanisms and their physiological function.

GRKs STRUCTURE AND DISTRIBUTION

GRKs comprise a family of seven mammalian serine/threonine protein kinases that phosphorylate and regulate agonist-occupied or constitutively active GPCR[1].There are three sub-groups within the GRK family. GRK1 (rhodopsin kinase) and GRK7 (cone opsin kinase) form one distinct sub-group that is only found in retinal cells. The non-visual GRKs divide into two sub-groups: the GRK2 subfamily, consisting of GRK2 (b-ARK1) and GRK3 (b-ARK2), and the GRK4 subfamily, consisting of GRK4, GRK5 and GRK6. GRK4 is predominantly found in the testes [2], to lesser extent, in some brain regions and the kidney[3,4], whereas GRK2, 3, 5 and 6 are widely expressed. In addition, the different GRKs are highly specific in their receptor preference[5,6].

The basic structure of non-visual GRK family members is similar, with a highly conserved central (263-266 amino acids) catalytic domain. The N-terminal 185-amino acid region displays considerable homology between individual GRKs. The similarity of the N-termini of GRKs has led to speculation that this region might be important in receptor recognition. All non-visual GRKs have a regulator of G-protein signaling (RGS) domain within the N-terminus region, which provides a potential mechanism by which GRKs might regulate GPCR signal transduction *via* phosphorylation-independent mechanisms. Indeed, growing evidence suggests that this could be the case for GRK2 and GRK3[7-9]. GRK4-6 possess a highly conserved binding site (amino acids 22-29 for GRK5) for phosphatidylinositol (4, 5)-bisphosphate [PtdIns (4, 5) P2], which is thought to enhance catalytic activity[8].The C-terminal of GRK2 and GRK3 is longer than that of the GRK4 subfamily, and contains a 125-amino acid pleckstrin homology (PH) domain. This domain glycine-rich β-globulin (Gbg) plays a role in targeting and translocation of these primarily cytosolic GRKs to membranes following GPCR activation[8]. More recently, a second binding site for Gbg-subunits has been identified within the first 53 amino acids of GRK2[9], which suggests that either the N- or the C-terminal regions might be sufficient to allow GRK2 targeting to the membrane. GRK4 and GRK6 are post-translationally palmitoylated at one or more cysteine

residues clustered within the last 15-20 amino acids of the C-terminus, leading to an exclusive membrane-associated localization[8]. GRK5 is also predominantly membrane-associated, and in this case localization is not achieved through lipidation but instead through the PtdIns (4, 5) P2 binding domain of the N-terminus and a polybasic region (amino acids 547-560) close to the C-terminus[11]. Further heterogeneity is possible within the GRK4 subfamily because both GRK4 and 6 are expressed in multiple splice variant forms[10]. Indeed, one splice variant of GRK4 lacks the N-terminal PtdIns (4, 5) P2 binding region, although the physiological significance of isoformic variation is not understood at present. GRK1 and 7 share many structural similarities with the non-visual GRKs, including an N-terminal RGS-like domain and central catalytic domain. Both GRK1 and 7 are membrane-associated; however, unlike GRK4 and 6, this association is via post-translational farnesylation at the C-terminal.

G-PROTEIN-COUPLED RECEPTOR ENDOCYTOSIS: DESENSITIZATION AND SIGNALING

GPCRs represent the largest family of transmembrane signaling molecules in the human genome. As such, they interact with numerous intracellular molecules, which can act either to propagate or curtail signaling from the receptor. Their primary mode of cellular activation occurs through heterotrimeric G proteins, which in turn can activate a wide spectrum of effector molecules, including phosphodiesterases, phospholipases, adenylyl cyclases and ion channels. In the immune system, triggering of GPCR is important for multiple activities, including cellular differentiation/activation, development of lymphoid tissue, and especially, for control of leukocyte chemotaxis. Active GPCRs are also the target of G-protein-coupled receptor kinases, which phosphorylate the receptors culminating in the binding of the protein arrestin. This results in rapid desensitization through inhibition of G protein binding, as well as novel mechanisms of cellular activation that involve the scaffolding of cellular kinases to GPCR-arrestin complexes. Arrestins can also serve to mediate the internalization of certain GPCR, a process which plays an important role in regulating cellular activity both by mediating long-term desensitization through down-regulation (degradation) of receptors and by recycling desensitized receptors back to the cell surface to initiate additional rounds of signaling. The mechanisms that regulate the subsequent intracellular trafficking of GPCR following internalization are largely unknown. Recently, however, it has become clear that the pattern of receptor phosphorylation and subsequent binding of arrestin play a critical role in the intracellular trafficking of internalized receptors, thereby dictating the ultimate fate of the receptor. In addition, arrestins have now been shown to be GPCRs that are capable of internalizing through arrestin-independent mechanisms[11].

GPCR responsiveness is determined by a tightly regulated balance among receptor signaling, desensitization, and resensitization. Receptor desensitization, the waning of GPCR responsiveness to the agonist with time, is an important, physiological "feedback" mechanism that protects against acute and chronic receptor over-stimulation[6]. The protein families of GRKs and arrestins play a pivotal role in the process of desensitization of agonist-activated GPCR[15-17]. There are seven known GRK subtypes, of which four members are expressed ubiquitously (GRK2, 3, 5 and 6)[15,18]. In the arrestin family, two members are restricted to photoreceptors, whereas β-arrestin1 and β-arrestin2 are expressed ubiquitously[15]. Agonist-induced desensitization of GPCR occurs via a multistep process. First, GRKs phosphorylate the intracellular loops and/or carboxyl terminal tail of the receptor, a process that enhances the affinity of the receptor for binding of cytosolic arrestin proteins. Subsequent binding of phosphorylated receptors by arrestins sterically inhibits interaction of the receptor with the G protein. Thus, agonist-induced phosphorylation of GPCR by a GRK, followed by binding of arrestins, efficiently prevents further coupling of the receptor to its G protein, thereby reducing or preventing receptor signaling[12]. Finally, the GRK-arrestin system promotes clathrin-mediated internalization of inactivated receptors to endosomal compartments for subsequent degradation or resensitization[15-17,19].

It is notable that besides its role in desensitization, β-arrestin-mediated receptor internalization can also regulate signal transduction. The internalized GPCR-β-arrestin complex can form a signalosome that activates signaling proteins, such as ERK1/2, p38 MAPK, and JNK. In addition, arrestins act as scaffolds that connect activated GPCR with tyrosine kinase c-Src and the PI-3K-AKT and NF-κB pathways[13,14].

GRKs display activities well beyond their classical role in receptor phosphorylation as well. For example, GRKs have been shown to interact with PI-3Ks and a guanosinetriphosphatase (GTPase)-activating proteins, GIT1, which are involved in regulating receptor trafficking and signaling[15,16]. In addition, GRK2 interacts with a component of the MAPK pathway, as well as with the PI-3K substrate AKT[17,18]. Furthermore, GRK2 and 3 are well-known to bind the Gβγ subunit complex, a process that induces activation of these GRKs. Direct interaction of GRKs with G proteins is suggested by the presence of regulator of RGS-like domains (RH domains) in GRKs[7,22-24]. RGS proteins act as GTPase-activating proteins (GAPs), which induce hydrolysis of guanosine 5'-triphosphate (GTP) and thereby inactivation of GTP-bound Gβγ subunits[19,20]. Selective binding of activated Gαq (and Gα-11) to RH domains of GRK2 and GRK3 (but not to RH domains of GRK1 and 4) was found to selectively inhibit Gq signaling. However, as GRK2/3 were shown not to act as GAPs for Gq[21,27-29], the main role of RH domains in GRK2/3 appears to prevent activated Gq from interacting with downstream effector molecules (Figure 1).

GRKs and arrestins also interact with non-GPCR. For instance, GRKs and arrestins interact with transforming growth factor -β (TGF-β), epidermal growth factor (EGF), and insulin growth factor receptors[22-28]. In addition, β-arrestin was found to regulate activity of Notch, an important protein in neurogenesis, angiogenesis, and

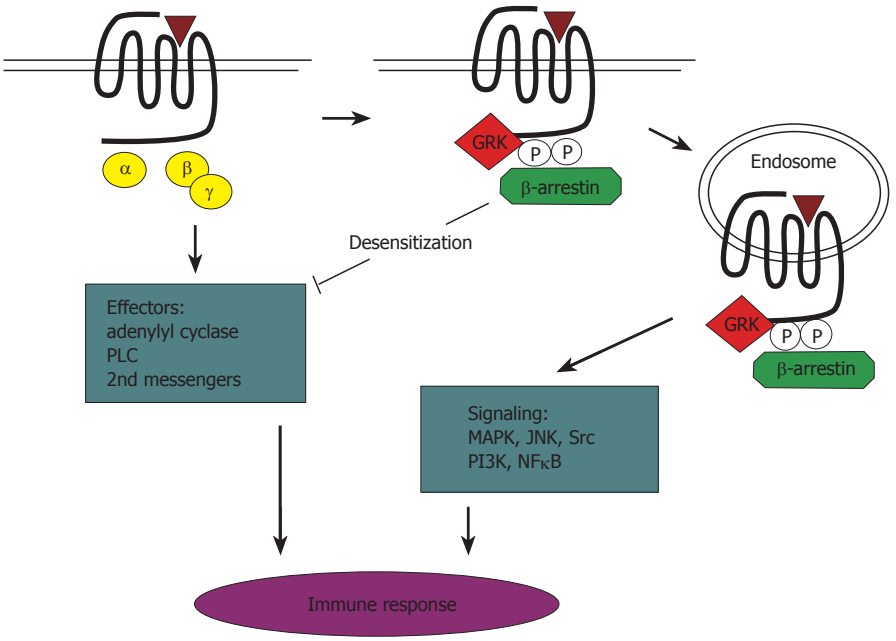

Figure 1 Schematic summary of the role of GRKs/arrestins in activation, signaling, and desensitization of GPCRs in the immune system. Agonist-activated GPCRs are phosphorylated rapidly by GRKs, leading to recruitment of arrestins. This process, called homologous desensitization, prevents further coupling of the receptor to its G protein, thereby reducing or preventing receptor signaling. In addition, GRKs and arrestins can also act as signal transducers in various signaling pathways. PLC: Phospholipase C.

lymphoid development[23]. GRKs and arrestins may directly affect functioning of these non-GPCR or modulate signaling of these receptors indirectly. Transactivation of growth factor receptors, such as the EGF receptor by GPCR, the β2- adrenergic receptor, CXCR4 chemokine receptor, or PGE2 receptor, has been described extensively[24-30]. Hence, GRK/arrestin-mediated regulation of GPCR signaling may indirectly affect signaling of such growth factor receptors. Interestingly, a recent study shows that formation of a PGE/β-arrestin-1/c-Src signaling complex in colorectal carcinoma cells is a crucial step in PGE2-mediated transactivation of the EGF receptor, indicating that arrestins also directly regulate the transactivation of a growth factor receptor by a GPCR agonist[39].

G-PROTEIN-COUPLED RECEPTOR INTERNALIZATION

An important aspect of GPCR activity and regulation is the internalization or sequestration of agonist-activated receptors into the intracellular membrane compartments of the cell. GPCR internalization has become the subject of intensive investigation over the past several years[25-34]. Consequently, a large volume of data has accumulated regarding the mechanisms regulating the endocytosis of a wide variety of different GPCRs. These studies have revealed GPCR domains involved in receptor endocytosis, some of the molecular intermediates that regulate GPCR endocytosis, and the potential for GPCR to internalize by multiple endocytic mechanisms. In addition, although the molecular mechanism involved in the initiation of GPCR endocytosis are best characterized for theβ2AR, recent studies using other GPCRs have revealed an important diversity in the patterns of GPCR endocytosis and intracellular trafficking.

The concept that GPCR are lost from the cell surface following agonist activation originated from the observation that β-adrenergic agonist treatment resulted in a loss of β-adrenergic receptor recognition sites on the surface of frog erythrocytes[26]. Subsequently, cell surface *versus* internalized β2AR binding sites were discriminated from one another either by differential sedimentation on a sucrose gradient or by using hydrophobic and hydrophilicβ-adrenergic ligands[26-27]. Internalized receptors were found in a "light vesicular" fraction, whereas cell surface receptors were found in a "heavy vesicular" plasma membrane fraction[26]. Similarly, internalized β2AR was accessible to hydrophobic, but not hydrophilic, adrenergic ligands[27]. More recently, the subcellular redistribution of cell surface β2AR in response to agonist activation was demonstrated by immunocytochemical staining of epitope-tagged receptors[28], as well as in real time microscopy in living cells using a green fluorescent protein (GFP)-taggedβ2AR[29]. Similar experiments have now been performed for several GPCR[30-41]. The rate at which GPCR internalize seems to be receptor-specific. For example, the A1 adenosine receptor internalizes quite slowly (t1/2 = 90 min) when compared with the A3 adenosine receptor (t1/2 = 19 min)[31]. These kinetic differences suggest that GPCR internalization can be mediated by multiple endocytic mechanisms and/or that structural heterogeneity between receptor subtypes modulates their relative affinities to bind endocytic adaptor.

G-PROTEIN-COUPLED RECEPTOR KINASES AND DISEASES

GPCRs form the largest family of cell surface receptors, and the defects in GRK function have the potential consequence to affect GPCR-stimulated biological responses in many pathological situations. Furthermore, the regulation of GRK levels in opiate addiction, cancers, psychiatric diseases, cystic fibrosis and cardiac diseases is discussed. Both transgenic mice and human pathologies have demonstrated the importance of GRKs in the

signaling pathways of rhodopsin, β-adrenergic and dopamine-1 receptors. The modulation of GRK activity in animal models of cardiac diseases can be effective to restore cardiac function in heart failure and opens a novel therapeutic strategy in diseases with GPCR dysregulation[32].

In human heart failure, impaired βAR signaling compromises cardiac sensitivity to inotropic stimulation[33]. The loss of receptor signaling is associated with an approximate three-fold elevation in myocardial βARK1 expression and GRK activity[34,35]. Myocardial ischemia and hypertension have also been associated with increased expression and activity of βARK1[36,37]. These aspects of human heart disease are similarly evident in animal models, where βARK1 levels are increased in cardiac hypertrophy[38] ischemia[43] and heart failure[39-43]. Given the variety of pathological insults represented in the animal models, βARK1 up-regulation appears to be an early common event in the pathogenesis of heart failure. In fact, βARK1 elevation often precedes the development of clinical heart failure and may represent a novel early marker for cardiac dysfunction. Like βARK1, GRK5 expression and activity are elevated in animal models[40-41], although its role in human heart failure remains unclear. In contrast, GRK3 expression is not increased in human heart failure[42]. At present it seems that for cardiovascular diseases, βAR polymorphisms do not play a role as disease-causing genes; however, they might be risk factors, might modify disease, and/or might influence progression of the disease. Furthermore, βAR polymorphisms might influence drug responses. Thus, evidence has accumulated that a βAR polymorphism (the Arg389GlyβAR) may affect the response to βAR-blocker treatment[42].

GRKs are implicated in the pathophysiology of human diseases, such as arterial hypertension, heart failure and rheumatoid arthritis. While GRK2 and 5 have been shown to be involved in the desensitization of the rat thyrotropin receptor (TSHR), their role in the pathophysiology of hyperfunctioning thyroid nodules (HTNs) is unknown. Therefore, scholars analyzed the expression pattern of the known GRKs in human thyroid tissue and investigated their function in the pathology of HTNs. The expression of different GRKs in human thyroid and HTNs was measured by Western blotting. The influence of GRK expression on TSHR function was analyzed by co-expression experiments in HEK 293 cells. Studies demonstrated that in addition to GRK2, 5 and 6, GRK 3 and 4 were also expressed in the human thyroid. GRK2, 3, 5 and 6 were able to desensitize TSHR *in vitro*. This GRK-induced desensitization is amplified by the additional over-expression of β-arrestin 1 or 2. No any mutation was found in the GRK2, 3 and 5 from 14 HTNs without TSHR mutations and Gsalpha mutations. The expression of GRK3 and 4 was increased in HTNs independently from the existence of TSHR mutations or Gsalpha mutations. In conclusion, the increased expression of GRK3 in HTNs and the ability of GRK3 to desensitize the TSHR *in vitro*, suggest a potential role for GRK3 as a negative feedback regulator for the constitutively activated cAMP pathway in HTNs[43].

CONCLUDING REMARKS

Much new information regarding the phosphorylation and regulation of GPCR by GRK2 and GRK3 and their role in GPCR signaling has been revealed during the past few years. More recent studies have started to indicate roles for GRK4, GRK5 and GRK6, both in transfected cell lines and in primary cells. However, it remains to be established whether the multiplicity of GRKs is related to the specificity or differential regulation of GPCR signaling or indeed other, yet to be defined, function. The association of particular GRKs within receptor signaling, trafficking and switching is a key area of current and future investigation

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S- Editor Liu Y **L- Editor** Kumar M **E- Editor** Liu WF

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World J Gastroenterol 2006 December 28; 12(48): 7758-7761
World Journal of Gastroenterology ISSN 1007-9327

ESOPHAGEAL CANCER

Glutathione-S-transferase M1 polymorphisms on the susceptibility to esophageal cancer among three Chinese minorities: Kazakh, Tajik and Uygur

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Received: 2006-9-28 Accepted: 2006-11-29

Abstract

AIM: To investigate the glutathione-S-transferase M1 (GSTM1) polymorphisms in three Chinese minorities, Kazakh, Uygur, and Tajik; and the pathological significance of GSTM1 polymorphisms in esophageal carcinogenesis in Kazakh.

METHODS: A total of 1121 blood samples (442 males and 679 females) were obtained from healthy Kazakh (654), Uygur (412) and Tajik (55). Primary esophageal squamous cell cancer (ESCC) tissues from Kazakh were obtained from 116 patients who underwent surgery. GSTM1 polymorphisms were analyzed by a combined approach of PCR and electrophoresis techniques.

RESULTS: GSTM1 null genotype was found in 62.63% Uygur, 50.91% Tajik and 47.40% Kazakh. A significantly higher frequency of GSTM1 null genotype in Uygur was observed compared with Kazakh (OR: 1.859, 95% CI: 1.445 -2.391, χ^2 = 23.71, P = 0.000). In addition, GSTM1 null genotype was found in 23.53% of well-differentiated ESCC in Kazakh, in 49.23% of poorly differentiated ESCC, with a significant difference (OR: 3.152, 95% CI: 1.403-7.080, χ^2 = 8.018, P = 0.007).

CONCLUSION: There is a marked difference in the frequency of common GSTM1 null genotype between Uygur and Kazakh. GSTM1 null genotype is associated with differentiation of ESCC in Kazakh.

Key words: Glutathione-S-transferase M1; Kazakh; Uygur; Tajik; Esophageal cancer

Lu XM, Yang T, Xu SY, Wen H, Wang X, Ren ZH, Zhang Y, Wang W. Glutathione-S-transferase M1 polymorphisms on the susceptibility to esophageal cancer among three Chinese minorities: Kazakh, Tajik and Uygur. *World J Gastroenterol* 2006; 12(48): 7758-7761

http://www.wjgnet.com/1007-9327/12/7758.asp

INTRODUCTION

Esophageal cancer (EC) is the sixth leading cause of cancer mortality worldwide[1]. The incidence of EC is highly variable in different populations, with more than a 50-fold difference between the high- and low-risk ethic groups[2,3]. For example, Turkomans in northeastern Iran are considered to be a very high-risk group, with age standardized prevalence (ASR) of over 100/100 000 for both men and women; whereas the prevalence of EC in pure Zoroastrian Persians in Iran and India are known to be considerably low, with ASRs of 3-7/100 000.

Epidemiological studies have identified several high EC incidence areas, such as the western and northern parts of China[4], certain areas of France and Brazil[5]. In Xinjiang Uygur Autonomous Region of China, there are thirteen minority ethnical groups (Uygur, Han, Hazakh, Tajik, Hui, Uzbek, Kerkez, Man, Mongolia, Tatar, Darur, Xibo, and Russian), who have lived there since ancient times. Uygur, Hazakh and Tajik are the major residents among those minorities with populations of 8 million, 2 million, and 40 thousand, respectively. Although they are all Muslims and have certain similarities in their life styles, the morbidities of EC among them are quite different. The incidence of EC in Kazakh is highest among all ethnics in Xinjiang, with an age-adjusted mortality of 90.7/100 000, significantly higher than that in Uygur (23.4/100 000) and almost 18-fold higher than that in Tajik (5.13/100 000)[4].

Glutathione S-transferases (GSTs) constitute a superfamily of ubiquitous multifunctional enzymes, which play a key role in cellular detoxification and protection of macromolecules from being attacked by reactive electrophiles[6]. GSTs catalyze the conjugation of tripeptide

glutathione (GSH) to a wide variety of exogenous and endogenous chemicals with electrophilic functional groups (e.g. products of oxidative stress, environmental pollutants, and carcinogens), thereby neutralizing their electrophilic sites, and rendering the products more water-soluble[7]. Based on sequence homology and immunological cross-reactivity, human cytosolic GSTs have been grouped into seven families, designated GST Alpha, Mu, Pi, Sigma, Omega, Theta, and Zeta[8]. The GSTs presumably arise from a single common ancestor and their substrate specificity and diversity have been reshaped by gene duplication, recombination and mutation.

There are marked intra- and inter-ethnic differences in the frequencies of common GST mutations[9,10]. For example, the distribution of GSTM1 genotype frequencies in Indian is significantly different from that in Chinese[11]. GSTM1 polymorphisms have been considered as a risk factor for EC development in a number of studies; however the overall results of such studies are inconsistent[12,13]. Up to date, data on genetic analysis of GSTM1 in Uygur, Tajik are lacking[14], and the correlation between GSTM1 polymorphisms and high incidence of EC in Kazakh has not been clarified.

The present study aimed to investigate the GSTM1 polymorphisms in healthy Kazakh, Uygur, and Tajik; and to explore the pathological significance of GSTM1 polymorphisms in esophageal carcinogenesis.

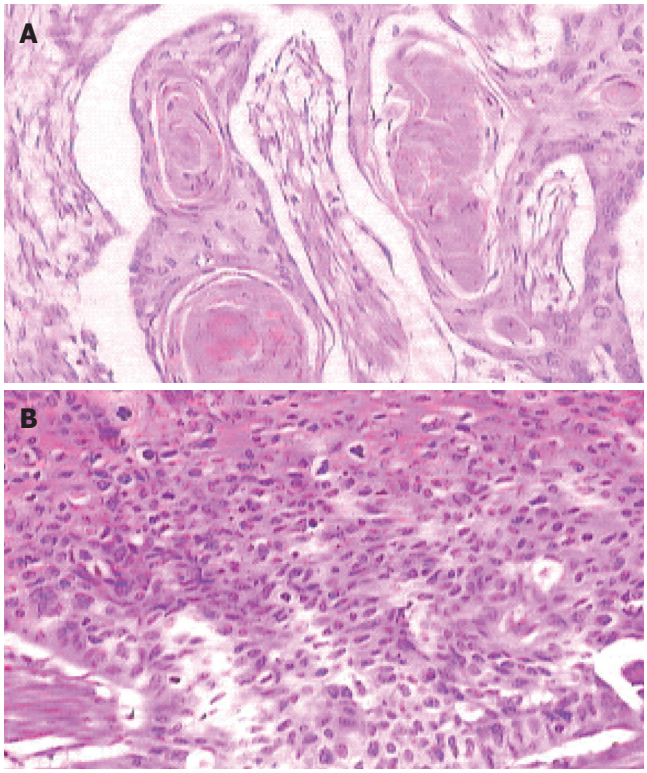

Figure 1 Histological types of primary ESCC (HE × 400). **A:** Well-differentiated ESCC; **B:** Poorly-differentiated ESCC.

MATERIALS AND METHODS

Blood and tissue

A total of 1121 blood samples was collected from healthy Kazakh (269 males and 385 females; age 35-65 years, mean 46.5 years), Uygur (146 males and 266 females; age 30-68 years, mean 45.5 years) and Tajik (27 males and 28 females; age 32-70 years, mean 47.5 years). All subjects from north-western of Xinjiang received clinical and biochemical assessments before entering this study and none of them has a clinical or family history of EC. Specimens of 116 primary EC tissues from Kazakh (84 males and 32 females; age 42-76 years, mean 55.5 years), with histological confirmation of primary ESCC, was recruited from two hospitals in Xinjiang from July 1999 to June 2004.

DNA extraction and GSTM1 genotyping

DNAs from healthy controls were extracted from peripheral leukocytes using the classical phenol-chloroform extraction method[15]. Genomic DNA in cancer tissue embedded by paraffin was prepared by proteinase K digestion and phenol/chloroform extraction, followed by ethanol precipitation, as described by Diffenbach[16]. The purity and concentration of DNA was examined by ultraviolet densitometry. GSTM1 genotyping for gene deletion was performed by PCR using primers 5'-GAAC TCCCTGAAAAGCTAAAGC-3' and 5'-GTTGGGCT CAAATATACGGTGG-3'[17], which produced a 219 bp product. At the same time, β-globin gene was amplified, resulting in a 350 bp product as an internal control. PCR was performed in a reaction mixture of 20 μL containing 100 ng sample DNA, 10 mmol/L Tris-HCl, 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$ pH 8.4, 0.1 mmol/L of each dNTP and 1.25 U Taq polymerase. After initial denaturation for 5 min at 94℃, 35 cycles were performed at 94℃ for 30 s (denaturation), at 63℃ for 30 s (annealing) and at 72℃ for 30 s (extension), followed by a final step for 5 min at 72℃. The amplified products were visualized by electrophoresis in ethidium-bromide-stained 1.5% agarose gel in TBE buffer. For genotype of GSTM1 deletion, no amplified product was observed except the band of β-globin gene.

Statistical analysis

Chi-square test was used to examine the correlation between the GSTM1 polymorphism among three healthy ethnics, and association of GSTM1 polymorphisms with differentiation of ESCC in Kazakh with SPSS software (11.0). Odds ratios (ORs) and 95% confidence intervals (CIs) of different variables among groups were calculated.

RESULTS

Histological types of primary ESCC

Histological confirmation of primary ESCC including well-differentiated and poorly-differentiated are shown in Figure 1.

GSTM1 genetic polymorphisms in ESCC of Kazakh

Figure 2 shows the PCR-amplified fragment of GSTM1. Genotype data for GSTM1 in the three ethnics are summarized in Table 1. The frequency of GSTM1 null mutation in Kazakh was significantly lower than that in Uygur (OR:1.859, 95% CI: 1.445-2.391, $\chi^2 = 23.71$, $P = 0.000$, $P < 0.05$). There was no significant difference

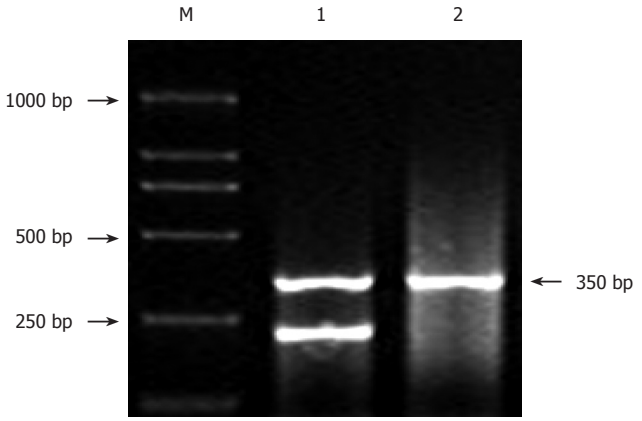

Figure 2 PCR of the GSTM1 genes. Lane M: DL 2000 DNA molecular weight marker; lane 1: GSTM1 genotype present; lane 2: homozygous deletion of GSTM1.

in the frequency of the GSTM1 null genotype between Uygur (62.63%) and Tajik (50.91%) ($\chi^2 = 2.804$, $P > 0.05$), and there was no significant difference between the Kazakh (47.40%) and Tajikic either ($\chi^2 = 0.250$, $P > 0.05$). In addition, no significant difference of GSTM1 null polymorphisms between the two genders of each ethnical group was observed.

There was a significant difference in the frequency of the GSTM1 null genotype between well-differentiation (high grade) (76.47%) and poor-differentiation (low grade) group (50.77%) of EC of Kazakh (OR 3.152, 95% CI 1.403-7.080, $\chi^2 = 8.018$, $P < 0.05$) (Table 2).

The odds ratio of GSTM1 null genotype of Kazakh people with lowly differentiated ESCC was 3.152-fold higher than those people with highly differentiated ESCC.

Table 1 Frequencies of GSTM1 polymorphisms in three ethnics

Ethnic	GSTM1		OR (95% CI)	*P*
	Null [*n* (%)]	Present [*n* (%)]		
Kazakh				
Male	130 (48.33)	139 (51.67)		
Female	180 (46.75)	205 (53.25)		
Total	310 (47.40)	344 (52.60)		
			[1]1.859 (1.445-2.391)	0.000
Uygur				
Male	99 (67.81)	47 (32.19)		
Female	159 (59.77)	107 (40.23)		
Total	258 (62.63)	154 (37.37)		
			[2]0.619 (0.352-1.809)	0.106
Tajik				
Male	13 (50.00)	13 (50.00)		
Female	15 (51.72)	14 (48.28)		
Total	28 (50.91)	27 (49.09)		
			[3]1.151 (0.664-1.996)	0.674

OR: odds ratio; CI: confidence interval. [1]Kazakh *vs* Uygur; [2]Uygur *vs* Tajik; [3]Kazakh *vs* Tajik.

Table 2 Correlation of clinicopathological grade of EC with GSTM1 genotypes in Kazakh

ESCC grade	GSTM1		OR (95% CI)	*P*
	Null [*n* (%)]	Present [*n* (%)]		
High	12 (23.53)	39 (76.47)		
Low	32 (49.23)	33 (50.77)	3.152 (1.403-7.080)	0.007

DISCUSSION

In this study, we investigated differences in the prevalence of GSTM1 null genotypes in three ethnical groups, Kazakh, Tajik and Uygur, in Xinjiang. As far as we know, we are the first to report the frequency of GSTM1 null genotype in Tajik. GSTM1 null genotype in Uygur in Xinjiang has a similar frequency when compared with Zoroastrians Iranian[18] and Han Chinese[19,20].

The study showed the association of GSTM1 null genotype with ESCC differentiation in Kazakh, suggesting the involvement of GSTM1 null genotype in the development of ESCC. Differences in the risk of EC development between high- and low-risk populations may partly be attributed to the genetic make-up of the populations, reflected by their different susceptibility to EC. GSTM1 encoding metabolic enzymes, the alteration in expression and function of which may increase or decrease carcinogen activation/detoxication, expressed as different phenotypes with different cancer risk[21-23]. Homozygous deletions of such genes, called GSTM1 null genotypes, result in the phenotype of no enzyme activity[24]. Individuals with null genotypes of GSTM1 are reported at high risk for developing several types of cancers, e.g. breast, lung, cervix[25-27] and bladder cancers[28-32]. However, the frequency of GSTM1 null genotype was low in Kazakh with high risk to EC, suggesting that the lack of the null allele or the other genes may play roles in carcinogenesis of ESCC by different mechanisms or via different pathways, from that of the reported breast, lung, cervix and bladder cancers. This large sample study on 654 of healthy Kazakh individuals and our previous genotyping results[14] have confirmed this contradictory finding of low frequency GSTM1 null genotype among Kazakh with a high susceptibility to ESCC.

In conclusion, there are different frequencies of GSTM1 null genotype among Uygur, Tajik and Kazakh, however, a significant difference is only observed between Uygur and Kazakh. The GSTM1 null genotype may play a role in the carcinogenesis and progress of ESCC.

ACKNOWLEDGMENTS

We thank those people who provided the blood samples of Kazakh, Tajik and Uygur individuals.

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S- Editor Liu Y **L- Editor** Zhu LH **E- Editor** Liu WF

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World J Gastroenterol 2006 December 28; 12(48): 7762-7767
World Journal of Gastroenterology ISSN 1007-9327

GASTRIC CANCER

Role of the HLA-DQ locus in the development of chronic gastritis and gastric carcinoma in Mexican patients

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Received: 2006-09-22 Accepted: 2006-11-20

Abstract

AIM: To determine the HLA-DQ locus in Mexican patients with Chronic gastritis and gastric adenocarcinoma.

METHODS: Oligotyping for HLA-DQ locus was performed in 45 Mexican patients with chronic gastritis and 13 Mexican patients with diffuse-type gastric adenocarcinoma, and was then compared with 99 clinically healthy unrelated individuals. *H pylori* infection and CagA status were assessed in patients by enzyme-linked immunosorbent assay (ELISA) method.

RESULTS: We found a significant increased frequency of HLA-DQB1*0401 allele in *H pylori*-positive patients with chronic gastritis when compared with healthy subjects [19 *vs* 0%, $P = 1 \times 10^{-7}$, odds ratio (OR) = 4.96; 95% confidence interval (95% CI), 3.87-6.35]. We also found a significant increased frequency of HLA-DQB1*0501 in patients with diffuse-type gastric carcinoma in comparison with healthy individuals ($P = 1 \times 10^{-6}$, OR = 13.07; 95% CI, 2.82-85.14).

CONCLUSION: HLA-DQ locus may play a different role in the development of *H pylori*-related chronic gastritis and diffuse-type gastric adenocarcinoma in the Mexican Mestizo population.

Key words: HLA-DQ; HLA-DQ5; HLA-DQB1*0501; *H pylori*; Chronic gastritis; Gastric cancer; Diffuse-type adenocarcinoma

Herrera-Goepfert R, Yamamoto-Furusho JK, Oñate-Ocaña LF, Camorlinga-Ponce M, Muñoz L, Ruiz-Morales JA, Vargas-Alarcón G, Granados J. Role of the HLA-DQ locus in the development of chronic gastritis and gastric carcinoma in Mexican patients. *World J Gastroenterol* 2006; 12(48): 7762-7767

http://www.wjgnet.com/1007-9327/12/7762.asp

INTRODUCTION

H pylori infection is, in addition to being the main etiologic agent for chronic gastritis, a major cause of peptic ulcer and gastric cancer[1]. In developing countries, prevalence of *H pylori* infection is > 80% among middle-aged adults, whereas in developed countries prevalence ranges from 20%-50%. Approximately 10%-15% of infected individuals will develop peptic disease and 3% a gastric neoplasm[2]. Therefore, *H pylori* infection is a necessary but not a sufficient cause of severe forms of gastric disease. In 1994 the International Agency for Research in Cancer (IARC), a branch of the World Health Organization (WHO), declared *H pylori* to be a Group 1 carcinogen, a definitive cause of cancer in humans[3]. Host genetic constitution is also thought to play a role in gastric carcinogenesis[4]. Among genetic factors, individual differences in inflammatory responses may protect or predispose to malignant transformation of the gastric mucosa. Human leukocyte antigens (HLA) class Ⅱ genes of the Major histocompatibility complex (MHC) are a group of highly polymorphic genes located in the short arm of chromosome 6 and are particularly important in controlling specific immune recognition[5]. HLA class Ⅱ antigens are capable of binding tumor peptides, and T-cell recognition of a combination of HLA class Ⅱ and bound tumor antigen may result in either induction of an effective anti-tumor immune re-

sponse or suppression of such immune response[6,7]. Moreover, adherence of *H pylori* to HLA class Ⅱ molecules expressed in gastric epithelial cells has been demonstrated[8].

Previous investigations have linked specific HLA-DQ alleles to gastric diseases, among others; Azuma *et al*[9] found increased susceptibility for *H pylori* infection in patients carrying the HLA-DQA1*0301 allele, whereas those displaying the HLA-DQA1*0102 allele were resistant to the infection; in other words, in Japan the HLA-DQA1*0102 allele has a lower frequency in *H pylori*-positive patients with atrophic gastritis compared with those with superficial gastritis and normal controls[10]. Conversely, the HLA-DQB1*0401 allele was found to be associated with atrophic gastritis in *H pylori*-infected patients[11]. On the other hand, the HLA-DQB1*0301 allele has been found more commonly in Caucasian patients with gastric adenocarcinoma[12]. The aim of this study was to investigate the relationship between HLA-DQ locus and presence of chronic gastritis and gastric adenocarcinoma in a Mexican population.

MATERIALS AND METHODS

Subjects

Forty five patients with chronic gastritis and 13 patients with diffuse-type gastric adenocarcinoma, all of them histologically confirmed, were studied. All patients were attended at the outpatient clinic of the Instituto Nacional de Cancerología (INCan) in Mexico City, because of gastric symptoms. A HLA-DQ database obtained from ninetynine healthy Mexican Mestizo asymptomatic subjects, without clinical evidence of chronic gastritis, peptic ulcer disease, gastric cancer, and personal or familiar history of autoimmune diseases was used for comparative purposes. Mexican Mestizo individuals included in the present study have a proportion of 56% Native American Indian genes, 40% White genes, and 4% Black genes[13]. Informed consent was obtained from all individuals considered in the present study.

Diagnosis of H pylori infection

H pylori status was assessed in patients by serologic analysis. Briefly, immunoglobulin G (IgG) antibodies against *H pylori* were tested in sera from 58 cases employing an enzyme-linked immunoabsorbent assay (ELISA) that was previously validated in Mexican population[14]. A pool of whole antigen preparation was obtained from sonicated preparations of three *H pylori* strains. Serum samples were diluted 1:1000, and 100-μL aliquots were plated. Next, a 1:1000 dilution of antihuman IgG monoclonal antibodies conjugated to alkaline phosphatase (Southern Biotech, Birmingham, AL, USA) was applied. A 1-mg/mL solution of p-nitrophenylphosphate was used as substrate and absorbance was read at 405 nm. All samples were analyzed by duplicate, the final value being the average of the two measurements.

ELISA for IgG anti-CagA

IgG antibodies for cytotoxin-associated gene A (CagA) protein were tested in patient sera utilizing an ELISA assay previously validated by our group[14]. A total of 0.1 μg/well of recombinant CagA antigen (Acambis, Cambridge, MA, USA) was used and serum at a 1:200 dilution was added. Next, a 1:1000 dilution of antihuman IgG monoclonal antibodies conjugated to alkaline phosphatase (Southern Biotech) was applied. A 1-mg/mL solution of p-nitrophenylphosphate was used as substrate and absorbance was read at 405 nm.

HLA-DQ typing

Genomic DNA was obtained from peripheral blood leukocytes and extracted by standard techniques[15,16].

Amplification of genomic DNA

HLA-DQA1 and -DQB1 typing were performed by a polymerase chain reaction (PCR) procedure using Taq DNA polymerase (Promega, Madison, WI, USA) and hybridization with PCR sequence-specific oligonucleotide probes (PCR-SSOP). Primers used for HLA-DQ amplification included DQAAMP-A, -B, DQBAMP-A, and -B. These were synthesized in a DNA-SM automated synthesizer (Beckman, Palo Alto, CA, USA). These typing techniques were approved by the 12th International Histocompatibility Workshop.

Dot blot hybridization

Five percent of the amplified DNA was denatured in 0.4 mol/L NaOH for 10 min, neutralized in 1 mol/L of ammonium acetate, and transferred to a Hybond-N membrane (Amersham, Bucks, UK). The filters were prehybridized at 42℃ for 30 min in a solution containing 6X SSPE (30X SSPE: 4.5 mol/L NaCl, 0.3 mol/L NaH_2PO4, 30 mmol/L EDTA, pH = 7.4), 5X Denhard solution (2% bovine serum albumin, 2% polyvinylpyrrolidone 40, 2% Ficoll 400), 0.1% Lauryl-sarcosine, and 0.02% SDS. Then, the oligonucleotide probes labeled with Digoxygenin dideoxy-Uridine-Triphosphate (Dig-11-ddUTP) were added and hybridized at 42ºC for 3 h. The filters were washed twice in 2X SSPE, 0.1% SDS at room temperature for 10 min, once in TMAC solution [50 mmol/L Tris-HCl (pH = 8.0), 3 mol/L tetramethylammonium chloride, 2 mmol/L EDTA, 0.1% SDS] at room temperature for 10 min, and twice at 60℃ for 10 min. Dots were revealed using the Dig Nucleic Acid Detection Kit (Boehringer Mannheim Biochemical, Mannheim, Germany).

Oligonucleotide probes

Information on the sequences and specificities of the DQA1 and -B1 oligonucleotides was gathered from the 12th International Histocompatibility Workshop. Oligonucleotide synthesis performed using the cyanoethyl phosphoramidite technique in a Beckman DNA-SM automated DNA synthesizer following the manufacturer's protocol.

Statistical analysis

Gene frequencies were compared using a 2 × 2 contingency table and χ^2 test. Odd ratios (OR) and 95% confidence intervals (95% CI) have been calculated for the disease in carriers of specific alleles; OR were not adjusted by gender or age. Comparisons of allele frequencies between sub-groups were carried out using the EPIINFO statistical package (Version 5.0, USD Incorporated 1990, Stone Mountain, GA, USA). All *P* values quoted were corrected

Table 1 HLA-DQB1 allele frequencies in Mexican patients with chronic gastritis according to *H pylori* status

DQB1*	*H pylori* + *n* = 48		*H pylori* - *n* = 32		Healthy *n* = 198	
	n	af	*n*	af	*n*	af
*0401	11	0.229[a,b]	2	0.062	0	0
*0301	10	0.208	5	0.156	34	0.171
*0302	7	0.145	11	0.343	48	0.242
*0501	5	0.104	1	0.031	12	0.060
*0201	3	0.062	4	0.125	33	0.166
*0304	2	0.034	0	0	1	0.005
*0602	1	0.017	1	0.031	15	0.075
*0601	1	0.017	3	0.093	0	0
*0603	1	0.017	0	0	4	0.020
*0604	1	0.017	0	0	3	0.015
*0303	1	0.017	1	0.031	0	0

af: Allele frequencies; [a]$P = 0.04$, *vs H pylori* -, OR = 4.46; 95% CI: 1.12-31.7; [b]$P = 1 \times 10^{-7}$, *vs* healthy individuals, OR = 6.5, 95% CI: 4.73-8.54.

Table 2 HLA-DQA1 allele frequencies in Mexican patients with *H pylori*-associated chronic gastritis according to CagA status

DQA1*	CagA + *n* = 28		CagA - *n* = 20		Healthy *n* = 198	
	n	af	*n*	af	*n*	af
*0501	15	0.535[a,b]	2	0.100	45	0.227
*0401	5	0.178	6	0.300	33	0.166
*0301	4	0.142	6	0.300	51	0.257
*0101	1	0.035	3	0.150	20	0.101
*0201	1	0.035	1	0.050	22	0.111
*0303	1	0.035	0	0	0	0
*0102	1	0.035	1	0.050	17	0.085
*0103	0	0	0	0	5	0.040
*0104	0	0	2	0.100[c]	0	0
*0105	0	0	1	0.050	0	0
*0503	0	0	1	0.038	0	0
*0601	0	0	1	0.038	0	0
*0302	0	0	1	0.038	0	0

af: Allele frequencies; [a]$P = 0.002$, *vs* CagA-, OR = 10.38, 95% CI: 1.76-79.51; [b]$P = 0.0005$, *vs* healthy individuals, OR = 3.92, 95% CI: 1.62-9.55; [c]$P = 0.008$, *vs* CagA+ and healthy individuals, OR = 12; 95% CI: 7.71-18.68.

by Bonferroni test for multiple comparisons taking into account the number of alleles studied. Statistical significance was considered as $P < 0.05$.

RESULTS

Subjects

Among patients with chronic gastritis, there were 35 female and ten male patients with a mean age of 56.3 years (range, 22-87 years). Thirteen patients with diffuse-type adenocarcinoma were also studied; there were eight women and five men with a mean age of 65.5 years (range, 41-90 years). Among patients suffering from chronic gastritis, 24 individuals were serologically positive for *H pylori* (17 females and seven males), while 14 patients were serologically positive for CagA (12 females and two males, respectively); five patients (four woman and one man) were eliminated because they were CagA-seropositive but *H pylori*-seronegative yielding thus a false-positive reaction, as previously stated[17]. Mean age of patients harboring *H pylori* infection was 58.9 years and for CagA-positive individuals, 56.7 years; mean age of *H pylori*-negative individuals was 53.2 years. Conversely, in the group of gastric carcinoma cases there were four patients with serologic evidence of *H pylori* infection (three women and one man), whereas solely one female patient was *H pylori* CagA-positive. Mean age of *H pylori*-positive patients was 74 years, whereas for *H pylori*-negative patients this was 61.7 years. CagA was positive only in one woman 57 years of age. Group of clinically healthy subjects no serologically-tested consisted of 47 women and 52 men, with a mean age of 33 years.

HLA genotyping in patients with chronic gastritis

HLA-DQA1 allele frequencies were distributed similarly between *H pylori*-positive and -negative patients with a diagnosis of chronic gastritis (data not shown).

In addition, regarding HLA-DQB1 locus a significant increased frequency of HLA-DQB1*0401 was observed in the *H pylori*-positive group compared with the *H pylori*-negative group and clinically healthy individuals (Table 1). A significantly increased frequency of the HLA-DQA1*0501 allele was found in the group of chronic gastritis and CagA-positive patients compared with CagA-negative patients and clinically healthy individuals. Moreover, DQA1*0104 allele frequency was increased in patients with chronic CagA-negative gastritis compared with patients with CagA-positive chronic gastritis and clinically healthy individuals (Table 2).

Table 3 shows an increased frequency of the HLA-DQB1*0501 and DQB1*0401 alleles in the group of patients with CagA-negative chronic gastritis compared with patients with CagA-positive chronic gastritis and clinically healthy subjects.

Haplotype analysis revealed significant increased frequency of HLA DQA1*0401-DQB1*0401 in *H pylori*-positive patients compared with clinically healthy subjects (Table 4).

HLA genotyping in patients with gastric diffuse-type adenocarcinoma

No significant differences were observed in the allele frequency of DQA1 locus between patients with diffuse-type gastric adenocarcinoma and clinically healthy individuals (data not shown). On the other hand, the HLA-DQB1 locus showed an increased frequency of the HLA DQB1*0501 allele in patients with gastric adenocarcinoma compared with clinically healthy individuals ($P = 1 \times 10^{-6}$, OR = 13.07; 95% CI, 2.82-85.14) but not when *H pylori*-positive and *H pylori*-negative subjects were compared ($P = 0.38$) (Table 5). In addition, HLA-DQB1*0501 allele frequency in *H pylori*-negative patients was also significant when compared with healthy subjects. No significant differences were found in the analysis between patients with gastric adenocarcinoma according to CagA status and clinically healthy individuals (data not shown). In addition, haplotype analysis did not show significant differences between HLA-DQA1-DQB1 haplotypes in patients with gastric diffuse-type adenocarcinoma and clinically healthy individuals (data not shown).

Table 3 HLA-DQB1 allele frequencies in Mexican patients with *H pylori*-associated chronic gastritis according to CagA status

DQB1*	CagA + *n* = 28		CagA - *n* = 20		Healthy *n* = 198	
	n	af	*n*	af	*n*	af
*0301	7	0.250	3	0.150	34	0.171
*0302	3	0.107	5	0.250	48	0.242
*0401	3	0.107	8	0.400[a,b]	0	0
*0201	2	0.071	1	0.050	33	0.166
*0304	2	0.071	0	0	0	0
*0501	0	0	5	0.250[c,d]	12	0.060
*0602	1	0.035	0	0	15	0.075
*0601	1	0.035	0	0	0	0
*0603	0	0	1	0.050	4	0.020
*0604	1	0.035	0	0	3	0.015
*0303	0	0	1	0.038	0	0

af: Allele frequencies; [a]$P = 1 \times 10^{-7}$, *vs* healthy individuals, OR = 17.5, 95% CI: 10.1–30.31; [b]$P = 0.01$, *vs* CagA + , OR= 5.67, 95% CI: 1.22-28.07; [c]$P = 0.03$, *vs* CagA +, OR = 9.0, 95% CI: 1.86-223.8; [d]$P = 0.01$, *vs* healthy individuals, OR = 5.17, 95% CI: 1.37-18.83.

Table 4 Haplotype allele frequencies in patients with chronic gastritis according to *H pylori* status

DQA1-DQB1*	*H pylori* + *n* = 48		*H pylori*- *n* = 32		Healthy *n* = 198	
	n	af	*n*	af	*n*	af
0401–0401	10	0.172[a,b]	1	0.031	0	0
0501–0301	10	0.172	3	0.093	27	0.136
0301–0302	11	0.189	10	0.312	48	0.242
0501–0201	8	0.137	2	0.062	10	0.050
0101–0501	5	0.086	0	0	10	0.050
0201–0201	3	0.051	0	0	22	0.111
0301–0401	2	0.034	1	0.031	0	0
0302-0302	1	0.017	0	0	0	0

af: Allele frequencies; [a]$P = 1 \times 10^{-7}$, *vs* healthy individuals, OR = 6.08, 95% CI: 4.56–8.10; [b]$P = 0.03$, *vs H pylori* -, OR = 7.15, 95% CI: 1.2-158.8.

Table 5 HLA-DQB1 allele frequencies in Mexican patients with gastric cancer according to *H pylori* status

DQB1*	*H pylori* + *n* = 8		*H pylori* - *n* = 18		Healthy *n* = 198	
	n	af	*n*	af	*n*	af
*0501	4	0.500[a]	5	0.277[b]	12	0.060
*0201	2	0.250	0	0	33	0.166
*0401	1	0.040	1	0.100	0	0
*0602	1	0.125	0	0	15	0.075
*0604	0	0	1	0.055	3	0.015
*0301	0	0.125	7	0.388	34	0.171
*0302	0	0.125	4	0.222	48	0.242

af: Allele frequencies; [a]$P = 0.001$, *vs* healthy individuals, OR = 15.5, 95% CI: 2.80-87.68; [b]$P = 0.007$, *vs* healthy individuals, OR = 5.96, 95% CI: 1.55-22.55.

DISCUSSION

Several previous studies have reported an association between HLA class II molecules and gastric diseases. In this study, we found significant increased frequencies of HLA-DQA1*0501 in patients with *H pylori* CagA-positive serology when compared with *H pylori* CagA-negative individuals as well as clinically healthy subjects, and HLA-DQA1*0104 in *H pylori* CagA-negative patients when compared with *H pylori* CagA-positive patients and clinically healthy individuals. Among patients harboring *H pylori*-associated gastritis, those who were CagA-negative showed a significant increased frequency of HLA-DQB1*0401 and HLA-DQB1*0501 alleles compared with CagA-positive patients and clinically healthy Mexican Mestizo individuals. HLA-DQA1*0401-HLA-DQB1*0401 haplotype showed to be a combination with higher susceptibility for *H pylori*-related gastritis. The finding of a high frequency of the HLA-DQB1*0601 allele in patients with chronic *H pylori*-negative gastritis emphasizes the participation of pathogenic mechanisms other than *H pylori* infection. This association has not been reported previously, and it is important to note that a larger sample size should be studied to maintain such an association.

Regarding patients harboring *H pylori*-associated gastritis, Sakai *et al*[11] also found an association between HLA-DQB1*0401 allele and presence of atrophic gastritis.

On the other hand, the HLA-DQA1*0501 allele was associated in patients with chronic *H pylori*-positive, CagA-positive gastritis. HLA-DQ5 has been also reported in association with atrophy and intestinal metaplasia of the gastric mucosa[18]. Other associations between HLA-DQA locus and gastric diseases have been described: Azuma *et al* found a protective effect of the HLA-DQA1*0102 allele against *H pylori* infection and intestinal-type adenocarcinoma[10], as well as a high susceptibility for *H pylori* gastritis and duodenal ulcer in patients carrying the HLA-DQA1*0301 allele[19].

Separately, Magnuson *et al*[20] found that HLA-DQA1*0102 was inversely associated with *H pylori*-seropositivity with no correspondence with a reduced risk for gastric cancer; this more notorious with diffuse-type carcinoma.

Moreover, Watanabe *et al*[21] have recently shown an increased allele frequency of HLA-DQB1*0401 in patients suffering from intestinal-type adenocarcinoma compared with individuals with *H pylori*-infected non-ulcer dyspepsia. In a Mexican study, Garza-González *et al*[22] concluded that HLA-DQA1*0503 allele could confer resistance to development of carcinoma and high-grade dysplasia of the stomach. Nevertheless, in our study we confirmed no protective effect of HLA-DQ alleles. We also found an association between HLA-DQB1*0501 and diffuse-type gastric adenocarcinoma as compared with clinically healthy individuals.

Interestingly, HLA-DQB1*0501 allele frequency was statistically significant only in patients with gastric carcinoma despite the fact that the majority of patients with gastric carcinoma were *H pylori*-negative and those who were infected, CagA-negative. This association was strong, considering the small number of cases under study; however, it is necessary to increase the sample size in order to confirm such an association. HLA class Ⅱ molecules are closely associated with gastric diseases, particularly the HLA-DQ locus.

Risk for gastric diseases among ethnic groups with different HLA class Ⅱ allele expression reflects several polymorphisms of this and other loci, as genes related

to mucosa protection (i.e. mucins, and trefoil peptides), inflammatory responses (i.e. interleukin-1β; interleukin-1 receptor antagonist, and tumor necrosis factor), and metabolic detoxifying enzymes (phase I enzymes like cytochrome P450 superfamily, and phase II enzymes like glutathione S- and N-acetyl transferases)[4]. The subtle mechanism by which such polymorphisms may drive the immune response and host susceptibility related with a particular stimuli is unclear; nevertheless, in this case, the participation of a unknown and as yet uncharacterized neighboring HLA class Ⅱ antigen could not be ruled out.

Oncogenes and tumor suppressor genes may also participate in several ways; for example, a 13Gly→Asp mutation of the K-ras oncogene has been related with improved prognosis in patients suffering from colorectal carcinoma; this is due to better recognition of partially overlapping epitopes with the 13Asp peptide and presented with HLA-DQ7 molecules by CD4+ T-lymphocyte clones[23].

In Caucasians, HLA-DQB1*0301 has been linked with gastric carcinoma[12], even in the absence of *H pylori* infection; however, this allele is also significantly frequent in patients with carcinoma of the cervix uteri[24] and melanoma[25]. It is noteworthy that the HLA-DQB1*0301 allele is common in healthy Mexican population (G Vargas-Alarcón, personal communication).

Moreover, Wu *et al*[26] reported lower seropositivity of *H pylori* and a higher ratio of diffuse/intestinal-type carcinoma in Taiwanese patients carrying the HLA-DQB1*0301 allele, whereas the HLA-DQB1*0602 allele was associated with susceptibility to proximal gastric cancer. The role of the HLA-DQB1 locus in gastric cancer development was also confirmed by Quintero *et al*[27], who found a significant association between the HLA-DQB1*0602 allele and CagA-positive status with distal gastric cancer in Spanish population. In a Chinese population, Li *et al*[28] found an increased risk for gastric cancer in patients carrying both the CW*03 and DRB1*01 alleles, particularly among those infected with *H pylori*.

Current evidence indicates that the majority of individuals harboring *H pylori* infection remain asymptomatic during their lifetime, with no clinical consequence from their infection. In a community-based seroepidemiologic study in Mexico[29], seropositivity for *H pylori* infection was 66%, and > 80% of adults were infected by age 25 years; seroprevalence remained nearly unchanged after the third decade of life, with an increment in seropositivity of < 0.5% per year in persons between 30 and 69 years. Taken together, these data suggest that risk for gastric diseases depends on factors other than *H pylori* infection and age.

According to histo-epidemiologic classification, gastric adenocarcinoma is divided into intestinal- and diffuse-type adenocarcinomas[30]. In intestinal-type adenocarcinoma, a multi-step process that includes gastritis, atrophy, and intestinal metaplasia of the gastric mucosa has been claimed as the initial event preceding the appearance of gastric carcinoma[31]. Intestinal-type adenocarcinoma, which is more frequent in the distal portion of the stomach, is related to a greater degree with *H pylori* CagA-positive infection[32]. In this case, the mechanism of neoplastic transformation could be mediated by translocation of CagA protein into the gastric cells through a type Ⅳ secretion system[33]. Diffuse-type adenocarcinoma has been also associated with *H pylori* infection, although there are controversial reports on this issue; prevalence of *H pylori* infection in gastric cancer series has been reported from 29% to 100%[3]; allele comparisons between diffuse- and intestinal-type adenocarcinoma are further warranted. Thus, we hypothesize that HLA-DQB1*0501 is associated with genetic susceptibility for developing diffuse-type gastric adenocarcinoma in Mexican Mestizo population regardless of *H pylori* status.

Interestingly, HLA-DQB1*0501 confers protection from malaria anemia and malaria reinfections in Gabonese children[34]. This association appears to be dependent on the cytokine profile, predominantly interferon-γ (INF-γ) production by T-cells and supports the notion that HLA can direct the immune response toward Th1 or Th2 phenotype[35].

In conclusion, our results, together with the body of evidence published in the literature, support that genetic constitution through HLA-DQ locus determines the mechanism of disease as well as clinical and pathologic outcomes, triggered by the interaction between environmental factors and the gastric milieu. In other words, immunogenetic background among different ethnicities is manifested as resistance or susceptibility to the development of chronic gastritis and gastric adenocarcinoma.

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COMMENTS

Background
Gastric cancer is multifactorial in origin; HLA genes confer susceptibility and resistance to diseases.

Research frontiers
HLA-DQ alleles are linked to gastric diseases; HLA-DQ locus drives different mechanisms of gastric disease.

Innovations and breakthroughs
HLA-DQB1*0501 is associated with diffuse type gastric carcinoma; HLA-DQB1*0601 is associated non-*Helicobacter pylori* gastritis.

Applications
Genotyping of HLA-DQ alleles is useful for determining individual susceptibility and/or resistance to gastric diseases; Knowing individual HLA constitution is useful for prevention, early detection and opportune therapeutics of gastric diseases, particularly, gastric cancer.

Terminology
Human leukocyte antigens (HLA) class II genes of the Major histocompatibility complex (MHC) are a group of highly polymorphic genes located in the short arm of chromosome 6, and are particularly important in controlling specific immune recognition; HLA class II antigens are capable of binding tumor peptides, and T-cell recognition of a combination of HLA class II and bound tumor antigen may result in either induction of an effective anti-tumor immune response or suppression of such immune response.

Peer review
The authors reported HLA-DQ locus may play a different role in the development of Helicobacter pylori-related chronic gastritis and diffuse-type gastric adenocarcinoma in the Mexican Mestizo population. Their results together with the body of evidence published in the literature support that genetic constitution through HLA-DQ locus determines the mechanism of disease as well as clinical and pathologic outcomes, triggered by the interaction between environmental factors and the gastric milieu. This is an interesting and important study.

S- Editor Liu Y **L- Editor** Alpini GD **E- Editor** Bi L

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World J Gastroenterol 2006 December 28; 12(48): 7768-7773
World Journal of Gastroenterology ISSN 1007-9327

VIRAL HEPATITIS

Usefulness of noninvasive transient elastography for assessment of liver fibrosis stage in chronic hepatitis C

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Received: 2006-08-21 Accepted: 2006-11-23

Abstract

AIM: To evaluate the method of noninvasive transient elastography for assessment of histological stage of liver fibrosis in patients with chronic hepatitis C (CHC).

METHODS: Two hundred and thirty-seven patients with CHC were included in this study. Liver biopsy was performed under ultrasonography on 217 of the patients, excluding twenty with clear clinical evidence of liver cirrhosis. Fifty subjects without liver disease were enrolled as a control group (stage 0). Twenty-five patients with sustained virological response (SVR) to interferon (IFN) therapy were also enrolled. These patients underwent liver biopsy before IFN therapy. Examination of liver stiffness (LS) was performed by elastography.

RESULTS: Medians (50% levels) of LS were 4.1 (3.5-4.9), 6.3 (4.8-8.5), 8.8 (6.8-12.0), 14.6 (10.5-18.6), and 22.2 (15.4-28.0), respectively, in the fibrosis stages 0-4 (P < 0.001). LS was significantly correlated with four serum fibrosis markers. LS values in patients with SVR were 3.8 (3.5-5.6), 5.2 (4.4-6.8), 6.8 (6.1-7.6), and 6.1 (3.6-7.9), respectively, in the fibrosis stages 1-4. In all stages, LS for patients with SVR was significantly lower than that for patients who did not undergo IFN therapy. LS was significantly correlated with serum concentrations of hyaluronic acid, type Ⅳ collagen, type Ⅳ collagen 7S, and type Ⅲ procollagen N peptide.

CONCLUSION: LS correlated well with the histological stage of fibrosis. Changes in liver fibrosis stage may thus be estimated noninvasively using transient elastography.

Key words: Hepatitis C virus; Echography; Fibrosis; Stiffness; Interferon

Takeda T, Yasuda T, Nakayama Y, Nakaya M, Kimura M, Yamashita M, Sawada A, Abo K, Takeda S, Sakaguchi H, Shiomi S, Asai H, Seki S. Usefulness of noninvasive transient elastography for assessment of liver fibrosis stage in chronic hepatitis C. *World J Gastroenterol* 2006; 12(48): 7768-7773

http://www.wjgnet.com/1007-9327/12/7768.asp

INTRODUCTION

Hepatic fibrosis deeply involves in the advance of stage of chronic hepatitis C (CHC), eventually leading to liver cirrhosis. In addition, the incidence of hepatocellular carcinoma (HCC) increases as the stage of fibrosis associated with CHC progresses[1]. It has been reported that patients with CHC with low-stage fibrosis respond better to interferon (IFN) therapy than those with higher-stage fibrosis[2]. Accordingly, evaluation of the stage of liver fibrosis is important when treating CHC. Although liver biopsy has been considered a gold standard for evaluation of liver fibrosis stage, it is invasive, stressful and is sometimes refused by patients or causes complications. In addition, liver biopsy can have life-threatening complications[3-5]. For these reasons, it is not possible to perform liver biopsy for all patients with CHC. Furthermore, the tissue samples obtained by needle biopsy are sometimes inadequate in quantity for accurate diagnosis[6,7]. Ratings based on examination of the liver tissue specimens may vary even among the specialists in pathology[8]. In addition, staging of fibrosis based on liver tissue specimens allows only step-wise evaluation (rather than as a continuous variable). While several serum markers (hyaluronic acid, type Ⅳ collagen, type Ⅳ collagen 7S, and P-Ⅲ-P) are known to be useful for quantitative evaluation of the liver fibrosis, and are utilized for indirect testing using serum samples[9-12].

In general, liver stiffness (LS) increases as liver fibrosis progresses[13]. The FibroScan 502 (FS, EchoSens, Paris, France) for transient elastography is a new modality

developed for noninvasive evaluation of liver stiffness based on the following principle. Waves including elastic shear waves are emitted from the vibrator attached to the ultrasound transducer probe. Pulse-echo ultrasound acquisitions follow the shear waves, and the velocity of such waves through the liver can be determined. LS is calculated from the shear wave velocity using Young's modulus. Use of transient vibration presents several advantages. First, the transmitted elastic waves can be temporally separated from reflected elastic waves. Second, the acquisition time is short, enabling measurements to be made on moving organs. Transient elastography is thus well adapted to the study of the liver.

We examined the relationships between the liver fibrosis of patients with CHC and LS determined by FS as well as serum markers of fibrosis.

MATERIALS AND METHODS

Patients

For initial examination, 237 subjects with CHC managed as patients at the Osaka City University Hospital were enrolled. Of these patients, 214 underwent liver biopsy under ultrasonic guidance. All the 237 patients satisfied the following criteria: (1) they were HCV RNA-positive, (2) free of ascites, and (3) liver disease due to HBV or alcohol could be ruled out for them. The control group (n = 50) was composed of 30 healthy volunteers and 20 patients without hepatic diseases. The mean age was lower in the control group than in any group of patients with CHC (Table 1). The liver biopsy was carried out using a 15-gauge needle biopsy apparatus (Hakko Inc., Tokyo, Japan). The specimens were fixed, paraffin-embedded, and stained with hematoxylin and eosin (H&E). Histological evaluation of the liver specimens was performed by two senior pathologists specialized in liver pathology. Stage of fibrosis and grade of activity in the liver were estimated according to the classification of Desmet *et al*[14]. The interval between the liver biopsy and FS measurement ranged from 0 d (on the same day) to 6 mo. The stage of fibrosis in the control group was rated stage 0. Patients clinically diagnosed with liver cirrhosis on the basis of diagnostic imagings (including computed tomography and ultrasonography) and hematological tests (although liver biopsy had not been performed) were also included in the analysis as stage 4 patients. These patients were included since, if analysis had been confined to the patients who had undergone liver biopsy, results might have been biased, because the liver biopsy is seldom performed in patients with liver cirrhosis (since IFN therapy is not indicated for these patients) and is not possible in cirrhotic patients with thrombocytopenia. Furthermore, 25 patients with CHC exhibiting sustained viral response (both disappearance of serum HCV RNA and normalization of alanine aminotransferase in response to previous IFN therapy) were also included for the next stage of analysis. These 25 patients had undergone liver biopsy and histological evaluation of the liver tissue before IFN therapy (Table 2). The study protocol accorded with the Helsinki Declaration. Patients were enrolled after provision of informed consent.

Table 1 Characteristics of patients without SVR

Stage	0	1	2	3	4	*P*
n	50	113	68	18	38	
Age (yr)	46.2 ± 17.9	55.9 ± 12.8	59.7 ± 9.9	57.2 ± 11.6	65.0 ± 10.2	< 0.001
M/F	25/25	54/59	19/49	7/11	21/17	0.031
HCV serotype						
1		48	34	7	17	
2		23	12	1	3	
ND		42	22	10	18	NS
Histological -grading						
Minimal		57	11		2	
Mild		50	38	8	6	
Moderate		6	19	10	6	
Severe					1	
ND					23	

Results for age are given as mean ± SD deviation. SVR: sustained viral response; ND: not done.

Table 2 Characteristics of patients with SVR

Stage	1	2	3	4	*P*
n	9	8	3	5	
Age (yr)	60.0 ± 8.6	60.2 ± 10.3	66.0 ± 6.1	58.5 ± 8.1	NS
M/F	5/4	3/4	2/1	2/3	NS
Serotype					
1	4	3	1	2	
2	3	4	2	2	
ND	2	1		1	NS
Histological -grading					
Minimal	1			2	
Mild	3	4	1	1	
Moderate	4	3	2	1	
Severe	1	1	0	1	
Period from liver biopsy (d)	2141 (626-5667)	981 (282-2285)	2187 (1191-2946)	2240 (970-3074)	NS

Results for age are given as mean ± SD. SVR: sustained viral response; ND: not done.

Liver stiffness measurement

LS was measured by transient elastography using an FS. Briefly, the subject lay on the bed in the horizontally supine position, and a probe was placed on the skin above the right intercostal space. The velocity of shear waves, generated temporarily and passing though the liver, was combined with Young's modulus for automated calculation of elasticity[15]. The median of 10 consecutive measurements was used as the LS for a given subject, and expressed in units of kilopascals (kPa).

Serum markers of fibrosis

Blood for measurement of serum markers of fibrosis was sampled on the day of LS measurement. Of the markers, serum concentrations of type Ⅳ collagen (Ⅳ collagen) were measured with latex agglutination turbidimetry (PANASSAY Ⅳ C; Daiichi Fine Chemical Co., Ltd., Tokyo, Japan), with a normal range of not more than 150 ng/mL. Serum concentrations of type Ⅳ collagen 7S (Ⅳ collagen 7S) were measured by radioimmunoassay (type Ⅳ collagen-7S kit, Mitsubishi Kagaku Iatron Inc., Tokyo,

Table 3 Results of biochemical examination of patients

Stage	1	2	3	4	*P*
n	113	68	18	38	
Platelet ($\times 10^4$ μL)	18.0 ± 8.0	13.9 ± 6.6	12.1 ± 5.7	9.9 ± 5.6	< 0.001
AST (IU)	47.3 ± 31.9	51.6 ± 27.2	63.8 ± 33.4	95.9 ± 142.2	0.001
ALT (IU)	60.9 ± 59.3	61.0 ± 40.5	75.8 ± 51.0	86.8 ± 135.7	0.023
ALB (g/dL)	3.9 ± 1.0	3.6 ± 1.3	3.8 ± 1.0	3.3 ± 1.1	0.036
HA (ng/mL)[1]	56.7 ± 65.8	89.2 ± 112.0	113.8 ± 125.8	509.3 ± 404.7	< 0.001
Ⅳ collagen (ng/mL)[2]	121.0 ± 57.5	149.9 ± 67.9	176.8 ± 129.2	229.7 ± 108.8	0.020
Ⅳ collagen 7S (ng/mL)[2]	5.5 ± 1.8	6.0 ± 1.6	8.9 ± 2.9	8.0 ± 2.1	0.001
P-Ⅲ-P (U/mL)[2]	0.79 ± 0.19	0.88 ± 0.30	1.23 ± 0.60	1.02 ± 0.29	0.023

Results are given as mean ± SD. [1]HA was measured in 57 patients; [2]Ⅳ collagen, Ⅳcollagen 7S, and P-Ⅲ-P were measured in 52 patients. AST: asparate aminotransferase; ALT: alanine aminotransferase; ALB: albumin; HA: hyaluronic acid.

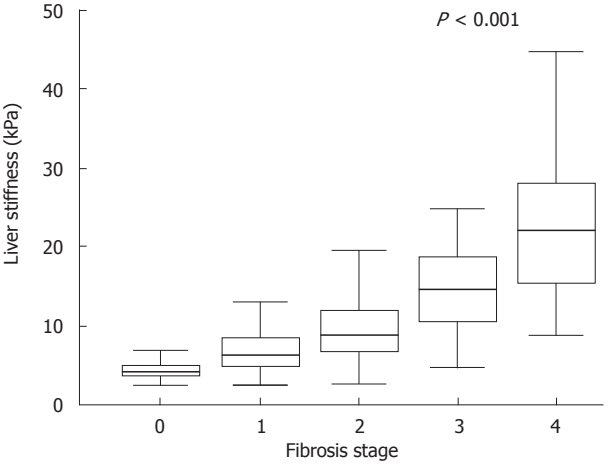

Figure 1 Liver stiffness measurements for each fibrosis stage. Fibrosis stage 0 is offered to the control group. The top and bottom of the boxes are the 1st and 3rd quartiles. The length of the box thus represents the interquartile range (IQR) within which 50% of values are located. The lines through the middle of the boxes represent the median. The error bars represent the minimum and maximum values (measurement range). Significant correlation was found between stage of fibrosis and liver stiffness ($P < 0.001$, Kruaskal-wallis test).

Japan), with a normal range of not more than 6 ng/mL. Serum concentrations of type Ⅲ procollagen N peptide (P-Ⅲ-P) were measured by radioimmunoassay (RIA-gnost PⅢ P c.t, Nihon Shering K.K., Osaka, Japan), with a normal range of 0.3-0.8 U/mL. Serum hyaluronic acid (HA) concentrations were measured by latex agglutination immunoturbidimetry (LPIA Ace HA, Fujirebio Inc., Tokyo Japan), with a normal range of not more than 50 ng/mL.

Statistical analysis

Biochemical data were expressed as mean ± SD. Elastography data were expressed as median values. Box plots were used to study the LS value distribution according to the stage of fibrosis. Differences in mean values were tested by one-way analysis of variance (ANOVA), followed by the Kruskal-Wallis test. The Mann-Whitney *U*-test was used to compare the data between the two groups. The χ^2 test was used to compare the distribution of individual variables among the patient groups. Correlations between two variables were examined using Spearman's correlation coefficient. Differences were considered statistically significant when *P* values were less than 0.05. All analyses were performed using SPSS 11.0J (SPSS Japan Inc. Tokyo, Japan).

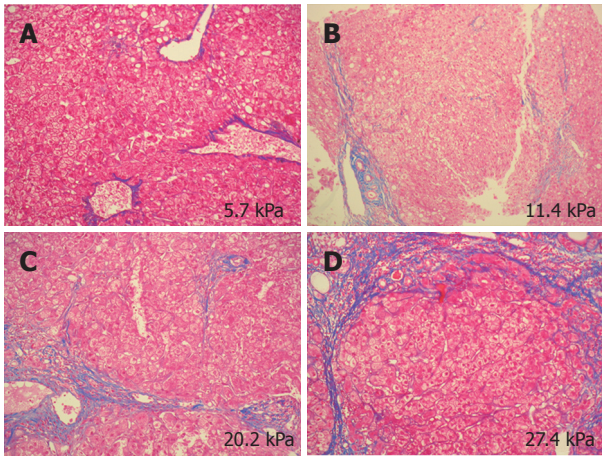

Figure 2 Azan-Mallory-stained liver tissue and liver stiffness measurement. **A** and **B:** stage 1 liver tissue samples, showing that the grade of fibrosis and elasticity was higher for **B** than for **A; C**: stage 3 liver tissue sample; **D**: stage 4 liver tissue sample. The elasticity thus increased as the fibrosis progressed.

RESULTS

Biochemistry

Table 3 shows the results of biochemical tests at each stage of CHC. Platelet count decreased as the stage progressed. Albumin level at stage 4 was significantly lower than at stage 1 ($P = 0.006$). Type Ⅳ collagen 7S level at stage 4 was significantly lower than at stage 1 ($P = 0.032$). However, HA level at stage 4 was significantly higher than at stage 1 ($P = 0.041$).

Relationship between the histological stage and the liver stiffness

Figure 1 shows LS determined with FS for the control group (stage 0) and serum HCV RNA-positive patients with CHC. Median LS (50% level) was 4.1 (3.5-4.9) at stage 0, 6.3 (4.8-8.5) at stage 1, 8.8 (6.8-12.0) at stage 2, 14.6 (10.5-18.6) at stage 3, and 22.2 (15.4-28.0) at stage 4 (Kruskal-Wallis test, $P < 0.001$). Mean LS differed significantly between each two of the five stages. The differences between the groups were as follows: stage 1 *versus* stages 2 ($P = 0.011$), 3 ($P < 0.001$), and 4 ($P < 0.001$); stage 2 *versus* stages 3 ($P = 0.013$) and 4 ($P < 0.001$); stage 3 *versus* stage 4 ($P = 0.004$). These differences were much superior to those obtained using the biochemical markers. Figure 2 shows the examples of findings for Azan-Mallory-stained liver tissue and LS. Samples A and B were classified as stage 1. However, degrees of fibrosis noted on microscopic examination differed between the two samples.

At each stage of fibrosis, LS was significantly lower in SVR than in HCV RNA-positive patients not treated with interferon (NT) (Figure 3). The median LS of SVR was 3.8

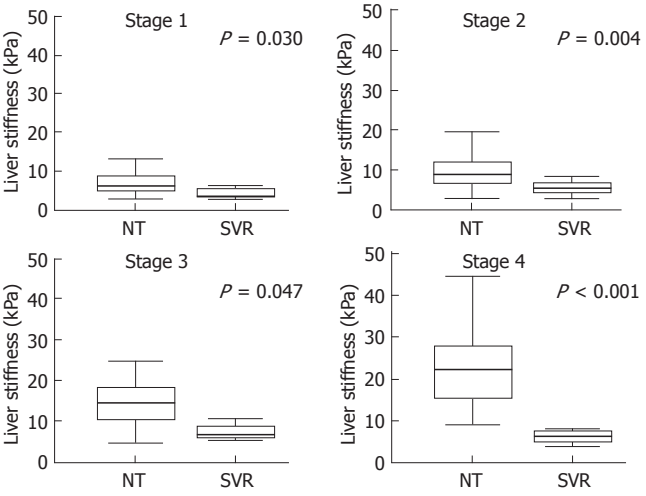

Figure 3 Liver stiffness measurements for each fibrosis stage in the patients not treated with IFN and the patients with sustained response for IFN therapy. Fibrosis stage 0 indicates the control group. The top and bottom of the boxes are the 1st and 3rd quartiles. The length of the box thus represents the interquartile range (IQR) within which 50% of values are located. The lines through the middle of the boxes represent the median. The error bars represent the minimum and maximum values (measurement range). In each stage, liver stiffness differed between NT and SVR. At each stage of fibrosis, elasticity was significantly lower in SVR than in HCV RNA-positive cases without IFN therapy (NT). NT: Patients without IFN therapy; SVR: Patients with sustained viral response to IFN therapy.

(3.5-5.6) ($P = 0.030$) at stage 1, 5.7 (4.4-6.8) ($P = 0.004$) at stage 2, 6.8 (6.1-7.6) ($P = 0.047$) at stage 3, and 6.1 (3.6-7.9) ($P = 0.001$) at stage 4 (compared with NT patients).

Correlations with serum markers of fibrosis

When correlations between the liver stiffness and serum markers of fibrosis were determined, the coefficient of correlation (r) was found to be 0.474 with HA ($n = 70$, $P < 0.001$), 0.581 with type Ⅳ collagen ($n = 70$, $P < 0.001$), 0.581 with type Ⅳ collagen 7S ($n = 69$, $P < 0.001$), and 0.233 with P-Ⅲ-P ($n = 70$, $P = 0.064$), respectively (Figure 4). Each of the four serum markers of fibrosis were significantly correlated with LS measured using FS.

DISCUSSION

HCC is the most frequent cause of death among patients with CHC. As liver fibrosis associated with CHC advances, HCC develops with high incidence[1]. It has been reported that the rate of viral eradication in response to IFN therapy is high in the patients with low-stage liver fibrosis[2,16]. Evaluation of the liver tissue is thus quite important when selecting a method of treatment and predicting prognosis for the individual patients with CHC. The stage of CHC is usually evaluated based on general assessment of hematological data and findings of diagnostic imagings. However, with these indirect tests, it is difficult to precisely determine the stage of liver fibrosis. Liver biopsy is a gold standard for direct evaluation of liver fibrosis. However, since it is invasive and stressful, and carries certain risks, it cannot be performed for all patients with chronic liver disease[17,18]. Furthermore, it is difficult to perform liver biopsy several times in the same patient. Regev *et al*[19] found that severity of fibrosis differed by

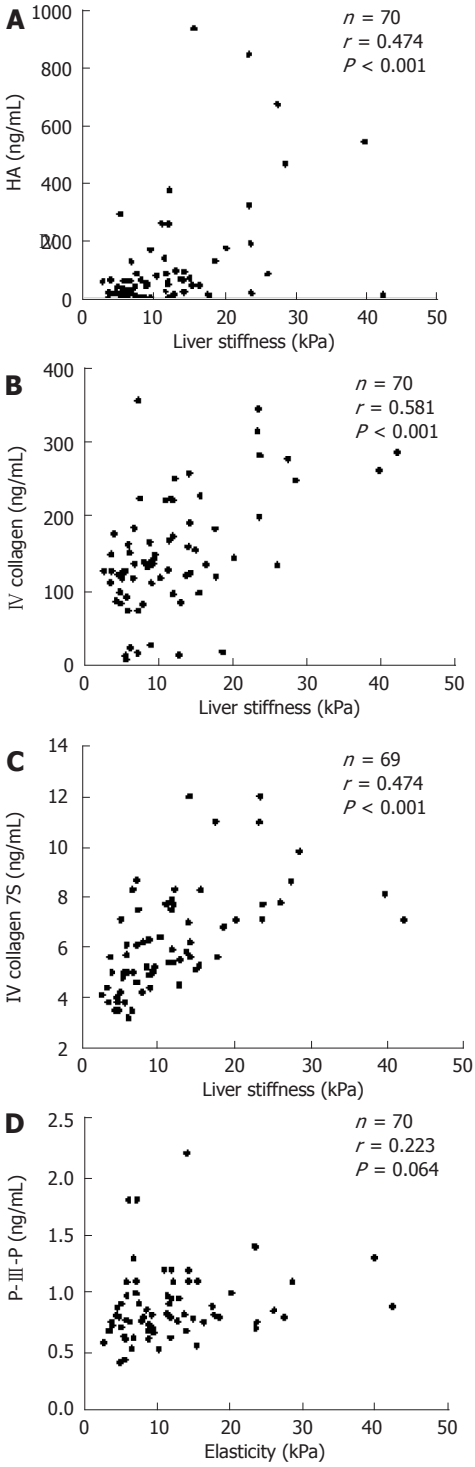

Figure 4 Correlation between liver stiffness and serum fibrosis markers. Correlations between elasticity measured by the Fibroscan502 and HA (**A**), type Ⅳ collagen (**B**), Ⅳ collagen 7S (**C**), and P-Ⅲ-P (**D**) are indicated. **HA: Hyaluronic acid; P-Ⅲ-P: Type Ⅲ procollagen N peptide.**

at least one stage between the right and left lobes of the liver in 41 of 124 (33%) patients studied. Bedossa *et al*[7] reported that accurate staging by the METAVIR fibrosis staging method was possible in only 75% of cases when liver biopsy specimens of at least 25 mm in length were used for evaluation. Siddique *et al*[20] found that degree of fibrosis differed by at least one stage in 45% of cases when tissue specimens collected from the same puncture site were evaluated. With liver biopsy, precise determination

of the stage of liver fibrosis is sometimes impossible, depending on the amount of tissue sample available. Furthermore, results of evaluation can differ among pathologists[21,22]. Correlations between fibrosis markers and the stage of fibrosis due to liver disease (including liver cirrhosis) have also been reported[9-12]. In the liver, HA is synthesized and secreted by fat-storing cells, which are liver-specific pericytes considered to be major contributors to liver fibrosis[23]. Elevated serum HA concentrations result not only from reduced catabolism of HA in the liver but also from excess hepatic production of HA. Type Ⅳ collagen is one of the major constituents of the basement membrane progressively laid down in fibrotic liver as a continuous subendothelial layer along the space of Disse. Appearance of type Ⅳ collagen 7S in serum is thought to be due primarily to degradation of existing basement membrane rather than newly synthesized type Ⅳ collagen[24]. Serum P-Ⅲ-P concentrations are thought to reflect mainly the degree of fibrosis and fibrogenic activity in chronic liver disease[25]. However, P-Ⅲ-P may also be derived from degradation of tissue type Ⅲ collagen still containing the amino-terminal peptide[26]. In addition, circulating P-Ⅲ-P is metabolized by the liver endothelial cells[24]. These factors may complicate interpretation of changes of serum P-Ⅲ-P, obscuring relationships between serum concentration and fibrogenic activity in the liver. Almost all human studies have used biopsy specimens for histological evaluation. In the present study, positive correlations between measurement with FS and serum markers of fibrosis were found.

Transient elastography using FS permits noninvasive measurement of LS from the body surface with a high degree of reproducibility. According to a recent report, LS determined with FS increased as the stage of liver fibrosis advanced in the patients with CHC. Liver biopsy permits only semi-quantitative evaluation of fibrosis, since degree of fibrosis can be expressed only in steps and not as a continuous variable. FS, on the other hand, may enable more quantitative evaluation of fibrosis. Saito *et al*[27] reported that platelet count correlated well with the stage of fibrosis, and that variation was large for platelet count but small for FS measurement. Measurement of liver stiffness with FS is superior to liver biopsy, in that the former does not cause pain or other adverse events and thus can be repeated. Furthermore, FS measurement features little inter-observer variation and is hence highly reproducible. Since LS determined with FS correlated positively with the stage of liver fibrosis, it should be possible to utilize FS to estimate the degree of liver fibrosis in patients with CHC[28,29]. Data from patients who underwent several liver biopsies indicate that liver fibrosis in the patients with CHC advances at a rate of approximately one stage per 10 years[30]. However, the rate of progression of fibrosis can vary depending on sex, alcohol consumption, and certain other factors[31]. In the present study, degree of liver fibrosis was significantly lower in the patients with CHC who had become SVR in response to IFN therapy than in HCV RNA-positive patients with CHC. This finding indicates that liver fibrosis was alleviated in the patients with CHC who had exhibited viral eradication in response to IFN therapy. A long-term prospective follow-up study of determination of liver stiffness by FS is now needed. The findings of the present study suggest that FS is a promising means of quantitative evaluation of the degree of liver fibrosis associated with CHC.

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S- Editor Liu Y **L- Editor** Kumar M **E- Editor** Liu WF

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World J Gastroenterol 2006 December 28; 12(48): 7774-7778
World Journal of Gastroenterology ISSN 1007-9327

Development of hepatitis C virus vaccine using hepatitis B core antigen as immuno-carrier

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Received: 2005-06-27 Accepted: 2005-11-18

Abstract

AIM: To develop hepatitis C virus (HCV) vaccine using HBcAg as the immuno-carrier to express HCV T epitope and to investigate its immunogenicity in mice.

METHODS: We constructed the plasmid pTrc-core^{NheI} using gene engineering technique, constructed the pcDNA3.1-core^{NheI}-GFP plasmid with GFP as the reporter gene, and transfected them into Hela cells. The expression of GFP was observed under confocal microscopy and the feasibility of using HBcAg as an immuno-carrier vaccine was studied. pTrc-core gene with a synthetic T epitope antigen gene of HCV (35-44aa) was fused and expressed in the plasmid pTrc-core-HCV (T). For the fusion of the HBcAg-T protein, sucrose, density gradient centrifugation was used, and its molecular weight and purity were analyzed by SDS-PAGE. Then balb/c mice were immunized by the plasmid with the HBcAg (expressed by pTrc-core) protein as control. The tumor regression potential was investigated in mice and evaluated at appropriate time. After three times of immunization, the peripheral blood and spleen of vaccinated mice were collected. HBcAb was detected by ELISA, and nonspecific T lymphocyte proliferation and response of splenocytes were respectively examined by MTT assay. T cell subset of blood and spleen were detected by FACS.

RESULTS: GFP was successfully expressed. Tumor regression trial showed that no tumor formation was found in the group receiving immunization, while tumor xenograft progression was not changed in the control group. Strong nonspecific lymphocyte proliferation response was induced. FACS also showed that the ratio of $CD8^+$ T cells in the experimental group was higher than the controls, but the serum HBcAb in experimental group was similar to the control.

CONCLUSION: HBcAg can be used as an immuno-carrier of vaccine, the fusion of HBcAg-T protein could induce stronger cellular immune responses and it might be a candidate for therapeutic vaccines specific for HCV.

Key words: Hepatitis C virus; Therapeutic vaccine; T epitope; Cellular immune responses

Chen JY, Li F. Development of hepatitis C virus vaccine using hepatitis B core antigen as immuno-carrier. *World J Gastroenterol* 2006; 12(48): 7774-7778

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INTRODUCTION

Hepatitis C virus (HCV) is a global public health problem, infecting 170 million people now, accounting for approximately 3% of the world population[1]. More than 50 million are infected with HCV in China, and the number has a tendency to rise. Persistent HCV infection has a high risk of progressing to chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma, usually more than a decade after initial infection[2]. Thus, it is important to develop adequate treatment for HCV infection. Polyethylene glycol plus Ribavirin treatment is effective, while new infections are continuously emerging from blood transfusion, needle sharing, close contact of HCV infected patients and other unidentified sources[3]. Thus to control the spread of HCV by developing new methods, such as therapeutic vaccines becomes an urgent task, especially in developing countries including China, where there is a large infected population.

In this study, we used HBcAg as immuno-carrier, inserted HCV T epitope into HBcAg el-loop, fused the protein particles of HBcAg-T *via* density gradient centrifugation, and immunized them in Balb/C mice with suitable dosage, then observed the immunogenicity of this fused protein in order to find a candidate for HCV therapeutic vaccine.

MATERIALS AND METHODS

Materials

The *E. coli* strain DH5α, JM109 and Hela cell line were conserved in our laboratory; Vector pTrc-core which carries a HBc gene was a kind gift of Dr. Li Jingli (Changchun, China); and Vector pcDNA-GFP was constructed in our laboratory which carries a GFP gene.

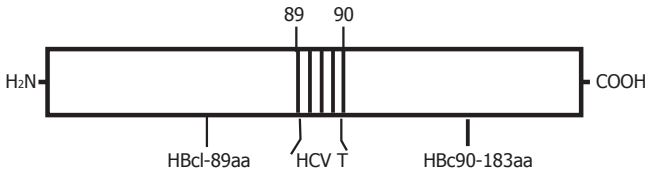

Figure 1 Hybrid HBcAg-T protein showing HCV T epitope (hatched box) inserted between HBcAg aa 79 and 80, a region located at the tip of core particle surface spikes.

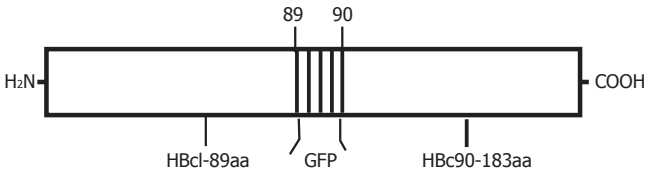

Figure 3 Hybrid HBcAg-GFP protein showing the GFP protein (hatched box) inserted between HBcAg aa 79 and 80.

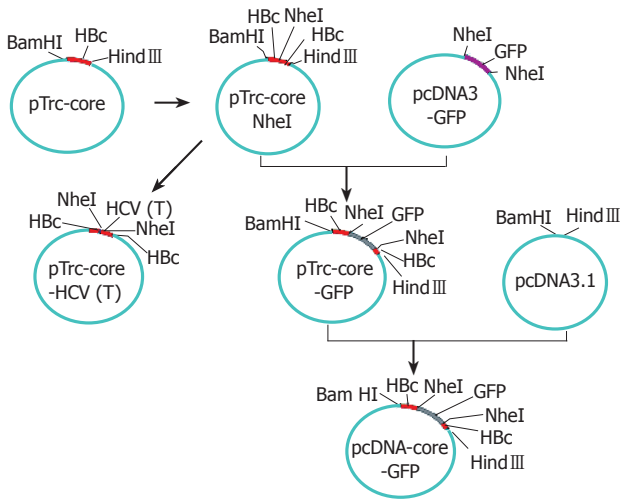

Figure 2 The construction of plasmids pTrc-core-HCV (T) and pcDNA3.1-core-GFP.

Restriction enzymes including NheI, HindIII and BamHI, T4 DNA ligase, Taq DNA polymerase, CIAP and RNase were purchased from TaKaRa Biotechnology (Dalian, China); Lipofectamine™ 2000 was purchased from Gibco Corporation; and other reagents were analytically pure reagents produced in China.

Construction of expression vectors

The HBcAg-T recombinant protein (Figure 1) was expressed in *E. coli* JM109 transfected with the expression of plasmid pTrc-core-HCV(T). This plasmid encodes a HBc gene (aa 1 to 183) with the HCV T epitope inserted into the HBcAg loop region between aa 79 and 80.

To construct this hybrid vaccine, a plasmid (pTrc-core^{NheI}) containing HBc gene was first digested at the NheI restriction sites, which had been strategically introduced into plasmid pTrc-core by PCR between the codons for P_{79} and A_{80} of the loop region. A synthetic double-stranded DNA fragment CTAGCgccgacctcatggggt acataccgctcgtcG encoding the sequence of HCV T epitope, modified by addition of 5' NheI and 3' NheI overhangs, was then inserted to yield plasmid pTrc-core-HCV(T) (Figure 2). This plasmid was used to direct the expression of a particle containing HCV T epitope. The positive clone of recombinant plasmid pTrc-core-HCV (T) was identified by PCR, restriction endonuclease cleavage and sequencing.

A second plasmid, pcDNA3.1-core-GFP (Figure 2), was used to direct the expression of a particle containing the GFP sequence fused in the 79-80aa loop region of the HBcAg (Figure 3). The plasmid pTrc-coreNheI was first digested with NheI restriction enzyme and dephosphorylated, then, a double stranded DNA fragment encoding the GFP sequence obtained from plasmid pcDNA3-GFP by the NheI restriction enzyme was inserted to produce plasmid pTrc-core -GFP. To yield the final expression vector pcDNA3.1-core-GFP, this plasmid pTrc-core-GFP was cut with BamHI and HindIII and then cloned into the pcDNA3.1 vector, which had been prepared with the same two restriction enzymes, then transfected the plasmid pcDNA3.1-core -GFP into the Hela cell line. Forty-eight hours after transfection, GFP fusion protein expression cells were directly observed under Olympus fluorescence microscope (Japan).

Expression and purification of recombinant particles

E. coli strain JM109 was transformed with either pTrc-core or pTrc-core-HCV (T) and selected on Luria-Bertani plates containing ampicillin (100 μg/mL). After 16-24 h of incubation at 37℃, a single colony was picked, expanded overnight, and used to inoculate a 500 mL culture (tryptone-yeast extract -NaCl [TYN] medium supplemented with 1 g glucose/L and 50 μg/mL ampicillin). After 16-20 h, cells were harvested by centrifugation. The pellets were resuspended in 5 mL of 25% sucrose in 50 mmol/L Tris pH8.0, added with 150 μL freshly prepared lysozyme (50 g/L), 100 μL of RNAse (10 g/L) and 100 μL of DNAse (10 g/L), mixed and incubated in a 37℃ water bath for 30 min. Samples were placed on ice for 5 min, added with 5 mL of lysis solution (10% Triton X-100, 0.4% sodium deoxycholate, 50 mmol/L Tris pH8.0 and 62.5 mmol/L EDTA) and incubated with occasional shaking at 37℃ for 30 min. The solution was incubated for a further 30 min at 37℃ and centrifuged at 4000 r/min for 15 min in a benchtop centrifuge. Supernatant was removed to a new tube. Lysate was vortexed at high speed for 30 s, and added with 2 mL of a 5 mol/L urea stock to create a 1 mol/L concentration. Three mL of this lysate was loaded onto the top of a sucrose gradient (60%, 50%, 40%, 30%, 20%) followed by density gradient centrifugation. After centrifugation at 4℃, 32 000 r/min for 22 h, samples (450 mL/tube) were collected to detect the ELISA response intensity using HBeAg diagnosis kit. Molecular weight and purity were identified by sodium dodecyl sulfate -polyacrylamide gel electrophoresis (SDS-PAGE), and ultraviolet spectrophotometer to measure its concentration. The purified proteins were stored at -20℃ prior to use.

Immunogenicity

BALB/c mice were obtained from Jilin University. Mice

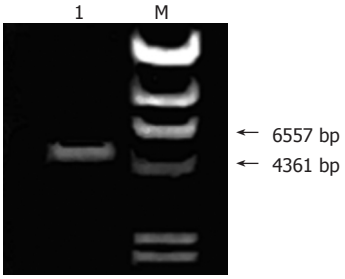

Figure 4 Plasmid pTrc-core^{NheI} identified by restriction endonuclease NheI cleavage. Land 1: pTrc-core^{NheI} digest; M: λ-Hind Ⅲ digest.

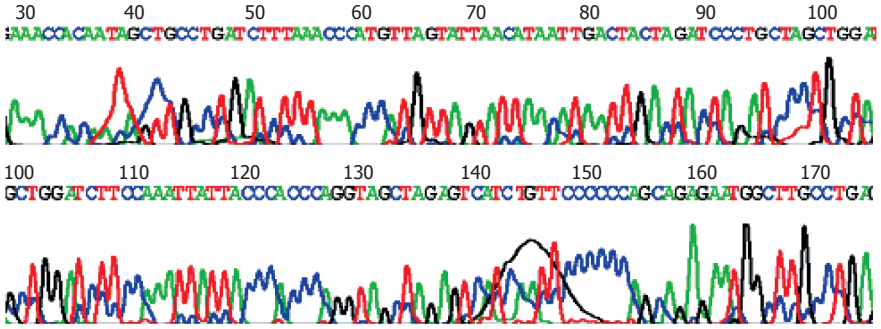

Figure 5 The sequencing of plasmid pTrc-core^{NheI} PCR product.

were immunized subcutaneouly with 2 μg HBcAg-T or HBcAg in Freund's incomplete adjuvant on d 0, 14, and 28. Fourteen days after the immunizing, half of the mice from each team were simultaneously inoculated with 200 μL (1×10^9/L) H22 cell, 10 d after the last protein immunizing, the mice were sacrificed to get the tumor mass. Serum samples were collected 30 d after immunizing. Five days after the booster inoculation, mice were sacrificed and spleen were removed for T-cell analysis. Untreated mice served as controls.

Serological assays

Anti-HBcAg antibody (HBcAb) was measured by ELISA using commercial kit (Rong Sheng, Shanghai, China), the endpoint titer was determined as the dilution of immune sera giving an optical density greater than the mean + 2 standard deviations obtained with normal sera.

T lymphocyte subgroup assays

The $CD3^+$, $CD4^+$ and $CD8^+$ T lymphocyte of blood and spleen were assayed by flow cytometry (Becton Dickson Company, USA).

Cellular proliferation assays

Triplicate wells containing spleen cells of naïve or immunized BALB/c mice were stimulated with HBcAg-T or HBcAg particles (2 g/L). Proliferation in 2-d cultures was measured by MTT to evaluate the cellular immune responses of the vaccinated animals, and results were expressed as stimulation indices (SI).

RESULTS

Identification of plasmid pTrc-core^{NheI}

On agarose gel electrophoresis, the product of pTrc-core^{NheI} digested by NheI formed a band of expected size of 5223 bp (Figure 4), The sequencing of plasmid pTrc-core^{NheI} PCR product was in agreement with expected (Figure 5). These proved that the NheI site was added successfully into the plasmid pTrc-core.

GFP expression

Under confocal microscopy, GFP was expressed successfully after plasmid pcDNA3.1-HBc-GFP was transfected into Hela cells (Figure 6). This suggested that GFP fragment was well exposed on the surface of chimeric VLPs, and the foreign gene inserted could be expressed correctly.

Identification of recombinant plasmid pTrc-core-HCV(T)

A 336 bp specific band was obtained by PCR amplification with pTrc-core-HCV (T) as template (Figure 7), and DNA sequencing confirmed that the sequence was completely concordant with what had been expected. These showed that the construction of plasmid pTrc-core-HCV(T) was successful. Sequencing result: (HCV T epitope underlined, NheI site italic) ctagacaccgcctcagctctgtatcgagaagccttagagtct cctgagcattgctcacctcaccatactgcactcaggcaagccattctctgctggggg gaattgatgacctagctacctgggtgggtaataatttggaagatcca*gctagc*gccga cctcatggggtacataccgctcgtc*gctagc*agggatctagtagtcaattatgttaatac taacatgggtttaaagacaggcaactattgtggtttcatatatcttgccttacttttgga agagagactgtacttgaatatttggtctctttcggagtgtggattcgcactcctcca.

Assay of recombination protein

Protein HBcAg-T and HBcAg were examined by SDS-PAGE. On the gel, a single protein band was displayed at about 2.1 kDa (Figure 8). It was consistent with the molecular weight of the two proteins. It suggested that the HBcAg-T and HBcAg was pure, and we could use them directly to immunize the animals.

Results of tumor regression trial

Four mice in HBcAg immunized group formed tumor masses (Figure 9), while none in the mice in HBcAg-T immunized group, the difference being obvious between the blank and HBcAg immunized groups ($P < 0.05$).

Results of HBcAb titer

ELISA showed that the difference of HBcAb titer between the control and HBcAg-T immunize groups was not significant (1:3880 *vs* 1:3900). This proved that T epitope inserted could not enhance the humoral immunity of mice obviously (Figure 10).

Results of $CD3^+$, $CD4^+$ and $CD8^+$ detection

FACS showed that the ratio of $CD8^+$ T cell in blood and spleen of HBcAg or HBcAg-T immunized mice was higher than the blank, and the ratio of HBcAg-T immunized group was the highest, showing that HBcAg-T could accelerate the proliferation of CTL, and induce stronger cellular immunity (Table 1).

Results of MTT

MTT showed that (Figure 11) HCV T epitope can accelerate unspecific T cell proliferative reaction ($P < 0.05$).

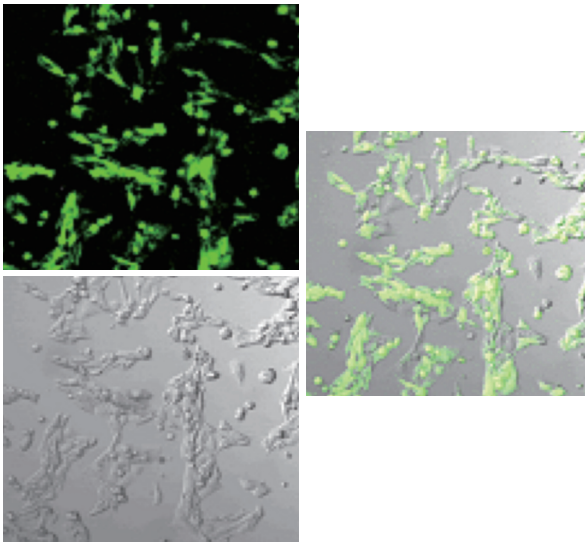

Figure 6 pcDNA3.1-HBc-GFP expression in Hela cell line (confocal microscopy, 300 ×).

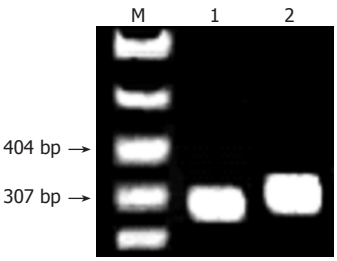

Figure 7 PCR products of pTrc-core-HCV(T). Lanes 1-2: pTrc-core^{NheI} or pTrc-core-HCV (T) PCR product; M: pBR322 DNA-Msp I digest.

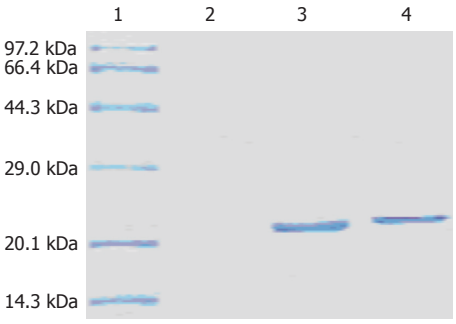

Figure 8 The results of HBcAg-T and HBcAg by SDS-PAGE. 1: Marker; 2: Negative; 3: HBcAg; 4: HBcAg-T.

Figure 9 Tumor regression trial in mice. 1: HBcAg immunized team; 2,3: Vacuity team; 4: HBcAg-T immunized team.

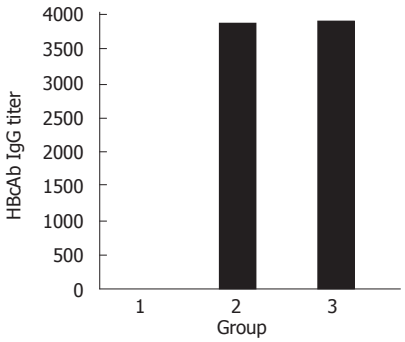

Figure 10 The titer of HBcAb. 1: Vacuity team; 2: HBcAg immunized team; 3: HBcAg-T immunized team.

DISCUSSION

The HCV is a highly variable virus, various attempts have been made to develop a vaccine against HCV infection, such as protein vaccine[4-6], oral vaccine[7] and the new RNAi technique[8]. Although some encouraging results were obtained, no effort on vaccine development has been successful. Recent studies about HCV epitopes showed that epitope vaccines might be a feasible strategy for HCV vaccine designs. Using the epitopes but not the whole HCV protein as the antigen could avoid HCV antigen protein to induce cancer, and its immunosuppressive action at the same time could refrain the patents from promoting HCV immune escape strain formation or aggravating infection degree induced by immune response for unsuitable sites. In this study, we chose a highly conservative T epitope (HLA-A_2 type) of HCV core region (35-44aa)[9] to design HCV therapeutic vaccine.

Only one epitope can not induce strong immune response because the immunogenicity is weak, we therefore used HBcAg as the immuno-carrier to develop HCV vaccines. Being a carrier of foreign epitopes, HBcAg has more advantages than other proposed particulate carriers. There are more powerful immunogenic epitopes[10], such as: Th epitopes which lie in 1-20aa, 28-47aa, 50-69aa, 81-105aa, 126-146aa and 141-165aa; HLA-A2,A31,A11 restricted CTL epitopes which lie in 18-27aa, 141-151aa and 88-96aa; and so on. So HBcAg carrier can provide a high level of T cell immunogenicity for the inserted HCV T epitope, its immune response may play an important role in HCV therapy. HBcAg can show exogenous epitopes with multicopy pattern, keep their original conformation, raise the epitope avidity, offer Th signal to exogenous epitopes and strikingly enhance immune responses. Experiments proved that HBC possesses Ti antigen property[11], which can induce immune response without adjuvant. Since Clarke[12] first reported on the virus-like particle (VLP) of HBcAg fusion in 1987, and constructed the first VLPs[13], HBcAg was often used as an immuno-carrier to develop immunogens and vaccines and had been used in many pathogens such as Hantaan virus, malarial parasite and HIV[14-16]. Now some investigators mentioned the features of HBcAg VLPs[17,18], and pointed out that the region between position 78 and 83 of HBcAg (maily immunodominant region, MIR) is surface accessible[19]. It was shown that HBcAg e1loop (78-83aa) was not necessary for assembling, without it HBcAg could auto-assemble into VLPs in vitro itself. In this study, we also showed that after GFP was introduced into this position, the assembly of HBcAg is unaffected and the inserted GFP could be exposed on the surface of VLPs. Thus, the position is an ideal site for HCV epitope inserting[20], and the foreign gene inserted here could be expressed correctly. Based on these findings, we inserted HCV T epitope into HBc e1loop, constructed fusion expression of plasmid

Table 1 Analysis of T cell subsets in peripheral blood and spleen

Group	Peripheral blood		Spleen	
	$CD4^+$	$CD8^+$	$CD4^+$	$CD8^+$
Control	15.25 ± 2.45	3.55 ± 1.65	5.02 ± 2.67	5.69 ± 1.71
HBcAg	27.54 ± 2.33	9.43 ± 1.56	27.71 ± 2.98	12.99 ± 1.35
HBcAg-T	25.14 ± 3.37	10.23 ± 1.85[b]	28.01 ± 2.05	15.01 ± 1.45[b]

[b]$P < 0.01$ *vs* HBcAg group.

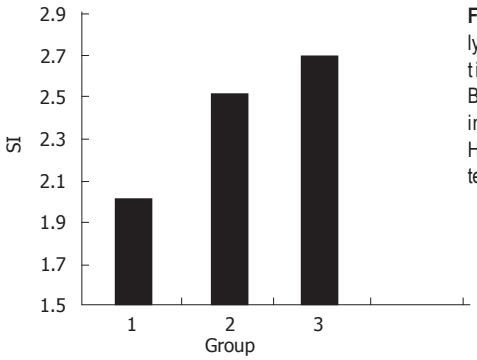

Figure 11 Nonspecific lymphocytes proliferative response. 1: Blank team; 2: HBcAg immunized team; 3: HBcAg-T immunized team.

pTrc-core-HCV (T), its expression product HBcAg-T was extracted, purified and quantitated, then immunized into Balb/c mice. We investigated its immunogenicity by some tests. Tumor regression trial showed that no tumor from the experimental group was found, being different from the control group. It means that the fusion antigens could stimulate mice to produce high-level CTL, which inhibits the tumor cells to survive; HBcAb was almost similar in the serum of the mice from HBcAg-T or HBcAg injected group, stronger nonspecific lymphocytes proliferation response was induced in experimental group; FACS also showed the ratio of $CD8^+$ T cell in the experimental group was higher than the control. All these findings demonstrated that HBcAg-T had induced stronger cellular immune response.

Since cytotoxic T lymphocytes (CTL) play a critical role in preventing the spread of HCV, and it has been suggested that cell-mediated immune responses played an important role in protection against HCV chronic infection[21], so vaccine-based HCV CTL induction could be a promising strategy to treat HCV-infected patients. In this study, the fused protein has been found to have the capacity to induce CTL in mice, so the success of this experiment undoubtedly could serve as a basis for developing HCV therapeutic vaccine.

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S- Editor Wang J **L- Editor** Ma JY **E- Editor** Liu WF

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World J Gastroenterol 2006 December 28; 12(48): 7779-7785
World Journal of Gastroenterology ISSN 1007-9327

CLINICAL RESEARCH

Validation of the Rockall scoring system for outcomes from non-variceal upper gastrointestinal bleeding in a Canadian setting

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Received: 2006-08-12 Accepted: 2006-11-30

Abstract

AIM: To validate the Rockall scoring system for predicting outcomes of rebleeding, and the need for a surgical procedure and death.

METHODS: We used data extracted from the Registry of Upper Gastrointestinal Bleeding and Endoscopy including information of 1869 patients with non-variceal upper gastrointestinal bleeding treated in Canadian hospitals. Risk scores were calculated and used to classify patients based on outcomes. For each outcome, we used χ^2 goodness-of-fit tests to assess the degree of calibration, and built receiver operating characteristic curves and calculated the area under the curve (AUC) to evaluate the discriminative ability of the scoring system.

RESULTS: For rebleeding, the χ^2 goodness-of-fit test indicated an acceptable fit for the model [χ^2 (8) = 12.83, P = 0.12]. For surgical procedures [χ^2 (8) = 5.3, P = 0.73] and death [χ^2 (8) = 3.78, P = 0.88], the tests showed solid correspondence between observed proportions and predicted probabilities. The AUC was 0.59 (95% CI: 0.55-0.62) for the outcome of rebleeding and 0.60 (95% CI: 0.54-0.67) for surgical procedures, representing a poor discriminative ability of the scoring system. For the outcome of death, the AUC was 0.73 (95% CI: 0.69-0.78), indicating an acceptable discriminative ability.

CONCLUSION: The Rockall scoring system provides an acceptable tool to predict death, but performs poorly for endpoints of rebleeding and surgical procedures.

Key words: Upper gastrointestinal bleeding; Nonvariceal; Predictors; Rockall; Outcomes

Enns RA, Gagnon YM, Barkun AN, Armstrong D, Gregor JC, Fedorak RN, RUGBE Investigators Group. Validation of the Rockall scoring system for outcomes from non-variceal upper gastrointestinal bleeding in a Canadian setting. *World J Gastroenterol* 2006; 12(48): 7779-7785

http://www.wjgnet.com/1007-9327/12/7779.asp

INTRODUCTION

Upper gastrointestinal (UGI) bleeding is a common disorder affecting over 100 per 100 000 population yearly[1-7]. The most common etiologies include peptic ulcer disease, mucosal erosive disease and variceal bleeding[8-12]. Because there is an increasing concern for cost-containment without sacrificing clinical outcomes[13-15], there is room to implement emergent care for UGI bleeding with appropriate early discharge for subjects at low risk of rebleeding or death[16-20]. Although endoscopic findings can identify individuals at a high risk of rebleeding, overall mortality is often reflective of other factors such as age and comorbid conditions. In an effort to risk-stratify subjects with UGI bleeding, numerous scoring systems have been developed to predict bleeding recurrences, and the need for surgical procedures and death[17,20-28].

One instrument designed for that purpose is the Rockall scoring system[27,28]. The Rockall system has been shown to represent an accurate and valid predictor of rebleeding and death, performing better in the latter than in the former[27-29]. Rockall scores are designed to combine information such as the subject's age, occurrence of

shock assessed from systolic blood pressure readings and pulse rate, presence and severity of comorbid conditions, diagnosis and endoscopic stigmata of recent bleeding. Summing up the different levels of a point grading system assigned to each of the components yields a subject's risk score bounded on a scale of 0 to 11, with 11 representing the highest risk. Results of previous investigations and validations of the scoring system have highlighted that those with a score of $\leqslant 2$ are associated with a very low rate of bleeding recurrences and death and, therefore, can be reasonably managed as outpatients. This has the potential to result in a more appropriate management of subjects' conditions based on their assessed risk of complications following the initial UGI bleeding. Further, managing low risk subjects as outpatients would free up scarce hospital resources for treating more serious cases.

Our objective was to validate the Rockall scoring system in the Canadian setting for the outcomes of rebleeding, the need for a surgical procedure and death, using data of 1869 patients with non-variceal UGI bleeding obtained from the registry of upper gastrointestinal bleeding and endoscopy (RUGBE)[30]. Additionally, we aimed to determine the mean length of hospital stay by levels of the Rockall score to compare current practice for subjects at a low risk of a serious event with an approach of managing their condition on an outpatient basis.

MATERIALS AND METHODS

The RUGBE initiative and data collection

A commercially available endoscopic reporting system (GI-Trac™, AD/MediTrac, Las Vegas, NV, USA) was linked to a project-specific patient registry. This software was distributed to 6 community and 12 university-affiliated health institutions across Canada, establishing a network, from which subjects were selected and source data collected. Research staff and monitors were trained at an initiation meeting and standardized definitions for all recorded variables were used. Information on all subjects was collected retrospectively from hospital records, denominalized and entered electronically in the reporting system. Data were then downloaded monthly (09/1999-12/2001) into the central repository and then, reviewed for internal logic and biological plausibility. All queries were resolved within one month of original data entry and 10% of all entries were audited quarterly for quality control.

Patient population

All subjects presenting with overt UGI bleeding or a history of hematemesis/coffee ground vomiting, melena, hematochezia, or a combination of any of the above within 24 h preceding admission were considered for the study. UGI bleeding was confirmed only if a member of the medical or nursing staff documented the presence of at least one of the following signs: (1) hematemesis; (2) melena; and/or, (3) bloody nasal gastric aspirates or black tarry material on rectal examination. Subjects were selected only if a UGI endoscopy was performed and a non-variceal source of bleeding was confirmed. A sequential time series sampling of eligible subjects was carried out at regular intervals to avoid a possible selection bias. An audit of all subjects presenting over a fixed time period at each institution was performed to further identify and prevent the possibility of a selection bias. The subset sampled constituted the entire dataset used in the study.

Study variables

Only the data for clinical and endoscopic variables necessary to build the Rockall risk scores and the outcome variables were extracted from the registry. Risk scores for each subject were calculated and used for risk stratification on the outcomes of rebleeding, the need for a surgical procedure and death. Standardized definitions for all outcomes were adopted according to adaptations of established definitions[31,32]. Continued bleeding following initial endoscopy was defined by the persistence of (a) spurting from an artery, (b) a bloody naso-gastric aspirate, (c) shock with a pulse greater than 100 beats per minute, a systolic blood pressure of below 100 mmHg, or both, or (d) the need for substantial replacement of blood and fluid volume (transfusion of greater than 3 units of blood within 4 h). Rebleeding was defined by recurrent vomiting of fresh blood, melena or both with either shock or a decrease in hemoglobin concentration of at least 2 g/L following initial successful treatment (modified from Daneshmend *et al*)[31,32]. Because the distinction is often blurred in practice, continued bleeding and rebleeding were subsequently combined within a single category termed 'rebleeding' for the purpose of this analysis.

Validation of the Rockall scoring system in the Canadian population

To validate the Rockall scoring system, we used χ^2 goodness-of-fit tests to assess the degree of calibration of each model (i.e. for outcomes of rebleeding, surgical procedures and death), and built receiver operating characteristic (ROC) curves based on a non-parametric technique as implemented in the statistical package STATA® for Window® for each outcome and calculated the area under the curve (AUC) along with 95% confidence intervals[33] to evaluate the discriminative ability of the scoring system. In our setting, a model has internal validity, or is well-calibrated, if it predicts the probability of experiencing an outcome that corresponds closely to the observed proportion of individuals with the outcome at each level of the Rockall risk score (i.e., from 0 to 11). The ROC curves plot the sensitivity of the Rockall score (true positive rate) versus 1-specificity (false positive rate) calculated for a series of different threshold values. The threshold values represent different levels of the Rockall scoring system, for which the rates of true positives (sensitivity) and true negatives (specificity) are calculated. The AUC is used to determine the ability of the scoring system to distinguish between individuals who experienced an outcome versus those who did not, over all possible threshold values. A test or risk scoring system with an AUC of 1 has a 100% sensitivity and 100% specificity, indicating that it would perfectly 'discriminate' between subjects experiencing the health event or not. A test with no better discriminative ability than what would otherwise be obtained by pure chance will have an AUC of 0.5, represented graphically by the

Table 1 Endoscopic findings in the registry of upper gastrointestinal bleeding and endoscopy (RUGBE)

Peptic ulcer disease	55.5%
Esophagitis	8.2%
Mallory Weiss	4.4%
Dieulafoy	2.5%
Other	29.4%

area under a 45 degree line. The accepted statistical rule of thumb is that a test with an AUC of less than 0.7 has a poor discriminative ability; an AUC between 0.7 and 0.8 provides acceptable discrimination and a test with an AUC above 0.8 is considered to have an excellent discriminative ability[34].

Because the ROC curves are plotted over all possible threshold values, it is possible to identify the optimal risk score cut-off value, at which the test is most accurate. For each ROC curve, we identified the optimal threshold of the Rockall score by: (1) determining the pair of sensitivity and specificity associated with the point geometrically closest to the upper left corner of the graph; (2) calculating the Youden index[35] (i.e., J = sensitivity + specificity - 1) for each score level. The cut-off level associated with the highest J coefficient is the one that minimizes the sum of false negatives and false positives.

Mean lengths of hospital stay per level of the Rockall score were also evaluated. To test for significant differences in the distribution of length of hospital stay between risk score levels we used Kruskal-Wallis non-parametric analysis because of the usual skewedness observed in the distribution of that variable.

RESULTS

The population of 1869 subjects included in RUGBE had a mean age of 66 years [standard deviation (SD): 16.9, range: 7-105], and 62% were males. Fifty-six percent were diagnosed with peptic ulcer disease as the primary etiology for UGI bleeding (Table 1). The mean Rockall score was 4.8 (SD: 1.9, range: 0-10). Overall, 13% of subjects would be considered at a low risk (i.e., Rockall score $\leqslant$ 2) of experiencing rebleeding or death, while 8% of the population was classified as at a high risk (i.e., Rockall score $\geqslant$ 8). The distribution of subjects across levels of the Rockall score is reported in Table 2, as well as the rates of events for the three outcomes of rebleeding, surgical procedures and deaths, and the mean lengths of hospital stay. The results showed that the rates of events typically increased with higher risk levels expressed by the Rockall score. A cutoff score of 8 or greater for high risk persons was based on the same value used in the original analysis by Rockall[35]. Alternatively, in Table 2 we also show the same results for categories with a score of 2 or less for low risk, 3 to 5 for moderate risk and 6 or higher for high risk. The mean length of hospital stay also followed a similar trend with increasing levels of the risk scores. The distribution for the length of hospital stay was quite skewed as shown by the summary statistics on median and interquartile range. Results from the Kruskal-Wallis test confirmed this finding by showing a significant difference in the distribution of length of hospital stay between score levels [χ^2 (7) = 78.7, P = 0.0001]. Figure 1 provides the graphical representation of the trends for the three outcomes and the length of hospital stay.

Calibration of the Rockall scoring system

In Figure 2A we show the comparison of observed proportions and predicted probabilities for the outcome of rebleeding. For most levels of the Rockall score, the predicted probability was slightly lower than the observed proportion of events. The result of the χ^2 goodness-of-fit test indicated an acceptable fit for the model, although calibration could be improved to show better internal validity [χ^2 (8) = 12.83, P = 0.12]. Our findings from the corresponding analyses for the outcomes of surgical procedures and death (Figure 2B and 2C) showed a good fit for the models and thereby, good calibration as the measure of internal validity. For surgical procedures and death, the χ^2 goodness-of-fit test indicated solid correspondence between observed proportions and predicted probabilities [χ^2 (8) = 5.3, P = 0.73 for surgical procedures; χ^2 (8) = 3.78, P = 0.88 for death].

Overall, the predicted probabilities were closer in value to the observed proportions in our subject population for the outcomes of surgical procedures and death. The correspondence for rebleeding was acceptable, but not as strong as that for the two previous outcomes.

Discriminative ability of the Rockall scoring system

The ability of the Rockall scoring system to distinguish between individuals experiencing the events of rebleeding, surgical procedures and death (ROC curve) is illustrated in Figures 3-5, respectively. For rebleeding, the AUC was 0.59 (95% CI: 0.55-0.62) indicating a poor discriminative ability, or external validity of the Rockall scoring system. A similar result was found for the outcome of surgical procedures with an AUC of 0.60 (95% CI: 0.54-0.67). For the outcome of death, the AUC was higher at 0.73 (95% CI: 0.69-0.78), interpreted as an acceptable discriminative ability of the risk scoring system. The optimal cutoff Rockall scores were 6 for surgical procedures and death and 7 for rebleeding. This means that, at these threshold levels, the sum of false negatives and false positives is minimized or that the accuracy of the scoring system is highest.

Overall, the internal and external validity of the Rockall risk scoring system was strongest for the outcome of death. With surgical procedures, the calibration achieved was high, but the scoring system had a poor discriminative ability. The level of calibration and discriminative ability were lowest for the outcome of rebleeding.

DISCUSSION

Several scoring systems have been developed to predict the clinical outcomes of gastrointestinal bleeding[17,21-25,27,28,35-40]. In 1987, Provenzale *et al*[28] tested various predictors of death from gastrointestinal bleeding and found that comorbid factors (i.e., liver and renal disease) and bleeding (i.e., hematochezia, short duration of bleeding, drop in

Table 2 Observed outcomes of subjects by Rockall score (% of total within score level)

Rockall risk score	Distribution of subjects	Rebleeding	Surgical procedure	Deaths	[b]Length of hospital stay (d)	
	n (%)	*n* (%)	*n* (%)	*n* (%)	Mean (SD)	Median (IQR)
⩽ 2	240 (13)	21 (8.8)	6 (2.5)	0 (0)	3.6 (3.5)	2.9 (1.1-4.7)
3	205 (11)	18 (8.8)	5 (2.4)	3 (1.5)	4.4 (5.9)	3 (2-5.25)
4	359 (19)	49 (13.6)	11 (3.1)	11 (3.1)	5.7 (5.7)	4 (2.3-7)
5	435 (23)	63 (14.5)	17 (3.9)	20 (4.6)	5.9 (6.9)	4 (2.3-7)
6	290 (16)	31 (10.7)	12 (4.1)	24 (8.3)	6.7 (7.9)	4.5 (2.3-8)
7	195 (10)	39 (20)	15 (7.7)	18 (9.2)	6.6 (6.6)	4 (2.3-9)
⩾ 8	145 (8)	37 (25.5)	9 (6.2)	24 (16.6)	7.4 (7.9)	5 (3-9)
Total	1869 (100)	258 (14)	75 (4.0)	100 (5.4)	5.7 (6.6)	4 (2-7)
Results for other risk score categories						
⩽ 2	240 (13)	21 (8.8)	6 (2.5)	0 (0)	3.6 (3.5)	2.9 (1.1-4.7)
3-5	999 (53)	130 (13)	33 (3.3)	34 (3.4)	5.6 (6.3)	4 (2-7)
⩾ 6	630 (34)	107 (17)	36 (5.7)	66 (10.5)	7.2 (7.7)	5 (3-9)

11 (0.59%) and 28 (1.5%) values were missing for outcomes of surgical procedure and death, respectively; IQR: Interquartile range (25% centile-75% centile); [b]P = 0.0001, comparison between risk score levels in distribution of length of hospital stay [Kruskal-Wallis test: χ^2 (7) = 78.7].

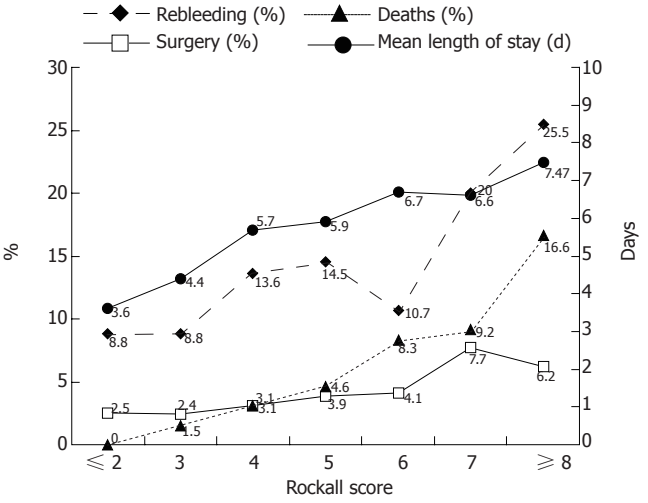

Figure 1 Clinical outcomes by level of the Rockall risk scoring system.

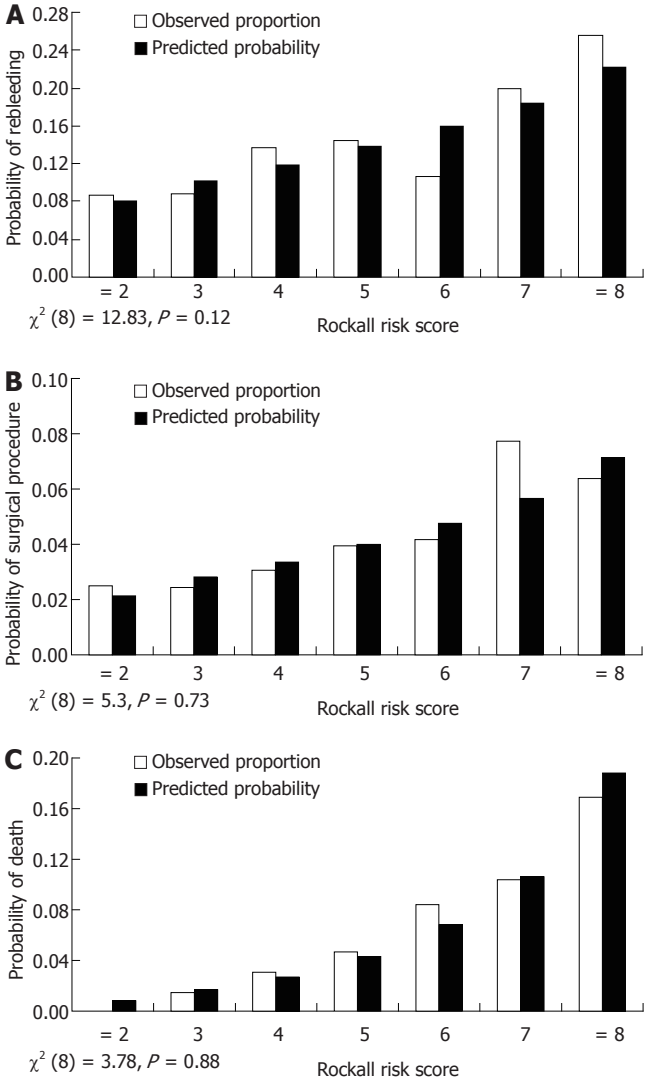

Figure 2 Predicted versus observed outcomes by Rockall risk core. **A**: Rebleeding; **B**: Surgical procedures; **C**: Death.

hematocrit of 5% and hypotension) were the most valid. Subsequently, several other risk scoring systems have been developed, with some of them validated in different patient populations[30,37,40,41].

Risk scores have been most commonly used as an aid to clinical decision-making to identify subjects who can be efficiently managed as outpatients, rather than being unnecessarily admitted for a prolonged hospital stay. Blatchford[17] and Rockall[27] have developed such scoring systems to forecast: (1) subjects' risk of rebleeding and death; (2) the need for early treatment of upper gastrointestinal bleeding. Although both scoring systems were designed for patients with UGI bleeding, the Blatchford scoring system does not incorporate information on endoscopic findings. This becomes an important limitation in circumstances where early endoscopic assessment is critical to optimal patient management. The Blatchford scoring system is still well-suited to the primary care setting when subjects need to be triaged to admission or outpatient management before an endoscopy is carried out.

When endoscopic information is available, the Rockall risk scoring system has been most widely applied to predict the risk of death and rebleeding. The system was originally

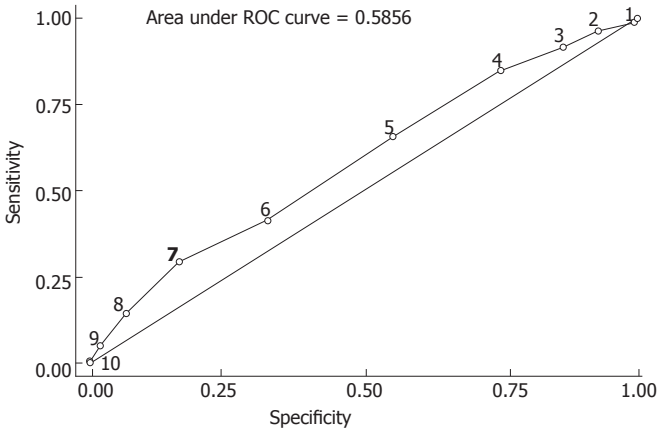

Figure 3 Receiver operating characteristic (ROC) curve for outcome of rebleeding. Numbers along the curve indicate Rockall risk score cutoff values; The optimal threshold is a Rockall score of 7 (in bold).

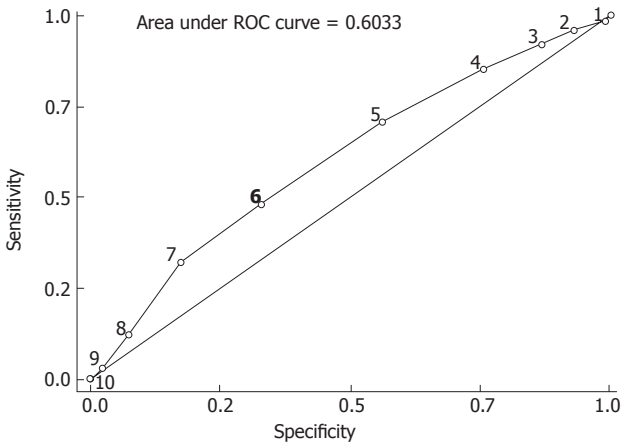

Figure 4 Receiver operating characteristic (ROC) curve for outcome of surgical procedure. Numbers along the curve indicate Rockall risk score cutoff values; The optimal threshold is a Rockall score of 6 (in bold).

developed to assess the risk of death, and its accuracy to forecast the risk of rebleeding has been shown to be relatively low in some validation studies[29]; however, in other studies the accuracy was relatively high. In two studies that assessed quality of care of a health care utilization[42-43], the risk of rebleeding appeared to correlate well with Rockall scores. In both studies there was considerable concern regarding excessive hospitalization of low risk Rockall patients since resources could be saved by early discharge.

Using data from a Canadian registry of subjects with non-variceal UGI bleeding, our objective was to test for the outcomes of rebleeding, surgical procedures and death: (1) the level of calibration of the Rockall scoring system as a measure of internal validity, and (2) the discriminative ability of the risk score for its generalizability to other populations. For that purpose, χ^2 goodness-of-fit tests for calibration and the area under the ROC curves for discriminative ability were used. Our results showed that the Rockall risk scoring system had acceptable performance for the outcome of death, but external validity and both internal and external validity were poor for surgical procedures and rebleeding, respectively. It is noteworthy that while subjects in the Vreeberg validation study[29] were from a different country (i.e. The Netherlands), the AUCs for the outcomes of death and rebleeding (0.73 and 0.61) resulted in almost identical numbers to ours (0.73 and 0.59). This adds weight to the conclusion that while acceptable to forecast the risk of death, the Rockall risk scoring system does not perform very well for the outcome of rebleeding. This study, performed in a Canadian setting, demonstrates that even with advanced endoscopic techniques, in a 'real-life' setting, the Rockall risk scoring system is acceptable for mortality prediction. Although the internal validity of the scoring system is high for the surgical procedures, its discriminative ability for this outcome is similar to that of rebleeding. We also found that the Rockall scoring system is in close agreement with length of hospital stay. This validation of the Rockall score is the first that has been done in a North American setting.

The Rockall risk scores are not widely used in Canada. However, it is clear that if endoscopic assessment could be expedient, a significant number of subjects (those

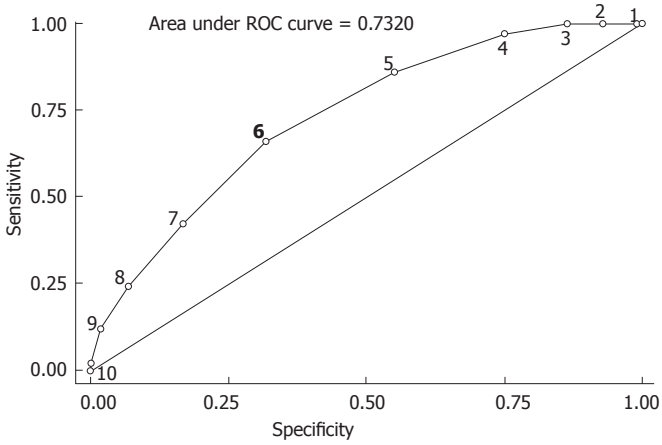

Figure 5 Receiver operating characteristic (ROC) curve for outcome of death. Numbers along the curve indicate Rockall risk score cutoff values; The optimal threshold is a Rockall score of 6 (in bold).

with Rockall scores of $\leqslant$ 2) at very low risk of death or rebleeding, might be discharged earlier and managed as outpatients since their risk of mortality is low. Although we have not demonstrated validity with rebleeding, in the setting of low Rockall scores it is clear that mortality is low (and in other studies rebleeding as well). Even if rebleeding does not correlate well, the risk of death is extremely low with low Rockall scores and this would likely still support early discharge.

It is unclear why such a low percentage of patients have low Rockall scores. One possible explanation is that in the Canadian medical system, access to rapid hospital admission is relatively difficult and it is conceivable that some low risk patients would be managed as outpatients without coming through a hospital setting and therefore might not be recorded in the RUGBE database.

One the one hand, when these patients are admitted to hospitals, it seems unusual that they are not quickly discharged. One reason why subjects with low Rockall scores are not discharged quickly is that endoscopic assessment is often delayed. In RUGBE, 76% of patients had been investigated at 24 h[30]. The lengthy assessment period may

contribute to the extended length of hospital stay.

On the other hand, subjects identified as high risk of death or rebleeding (Rockall score $\geqslant$ 8) may benefit from more intensive monitoring. This could be performed at an intensive care unit (ICU) or a 'step-down' unit rather than the usual medical/surgical wards. In Canada, the use of ICU beds for subjects with UGI bleeding has been shown to be less common than in some American centers[44]. In RUGBE, 22% of all patients were admitted to the ICU for investigation, therapy and monitoring[30] and even in the 145 patients with a Rockall score $\geqslant$ 8, only 52, or 36%, were sent to ICU. However, a large portion of them would have been classified as average risk and, therefore, unnecessarily monitored in the ICU. Other subjects with a high risk score were sent to medical/surgical wards instead of the ICU.

The strength of this study is in its 'real-life' evaluation of patients presenting to hospitals. The RUGBE database was a thorough one with internal validation that was collected retrospectively. This leads to the major weakness of the study, a retrospective evaluation. Although the RUGBE database was accurate, it was still retrospective with the associated weaknesses of a retrospective database. To device Rockall scores retrospectively will bring about occasional missing data and the inherent patient selection bias. Bias was limited in RUGBE (as best as possible) by having some sites receive virtually all of their non-variceal UGI bleeding patients over the specified time period.

This study has confirmed that the Rockall scoring system provides an acceptable tool to predict the risk of death, but performs poorly for endpoints of rebleeding and surgical procedures. Its cautious use for clinical decision-making purposes could still result in implementing more expedient care for low risk subjects, without sacrificing outcomes, and more efficient monitoring of high risk individuals.

ACKNOWLEDGMENTS

We wish to thank the The RUGBE investigators group, which includes: Alan Barkun, Carlo Fallone, and Gad Friedman, The McGill University Health Centre - the Montreal General and Royal Victoria Hospital sites, and the Sir Mortimer B Davis - Jewish General Hospital, Montréal, Québec; Raymond Lahaie, Georges Ghattas, and Judith Dorais, le centre hospitalier de l'université de Montréal, les pavillons hôpitaux St-Luc, Notre Dame, et Hôtel-Dieu, Montréal, Québec; Naoki Chiba, McMaster University, Hamilton, Ontario; David Armstrong and John Marshall, McMaster University & Hamilton Health Sciences, Hamilton, Ontario; Norman Marcon, St-Michael' s Hospital, Toronto, Ontario; Jonathon Love, the Queen Elizabeth II Health Sciences Centre, Halifax, Nova Scotia; Alan Cockeram, Saint John Regional Hospital, St John, New Brunswick; Franzjoseph Schweiger, Moncton Hospital, Moncton, New Brunswick; Jamie Gregor and John McDonald, London Health Sciences Centre, the University Hospital and Victoria Campuses, London, Ontario; Rob Enns, St Paul's Hospital, Vancouver, British Columbia; Richard Fedorak, Bob Bailey, and Connie Switzer, the University of Alberta, Royal Alexander, and Grey Nuns Hospitals, Edmonton, Alberta.

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S- Editor Wang GP **L- Editor** Zhu LH **E- Editor** Bi L

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World J Gastroenterol 2006 December 28; 12(48): 7786-7791
World Journal of Gastroenterology ISSN 1007-9327

CLINICAL RESEARCH

Factors influencing health-related quality of life in chronic liver disease

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Received: 2006-10-21 Accepted: 2006-11-23

Abstract

AIM: To investigate the factors contributing to health-related quality of life (HRQL) in chronic liver disease (CLD).

METHODS: Patients with CLD and age- and sex-matched normal subjects performed the validated Thai versions of the short-form 36 (SF-36) by health survey and chronic liver disease questionnaire (CLDQ). Stepwise multiple regression analysis was used to assess the impact of disease severity, demography, causes of CLD, socioeconomic factors, and self-rating health perception on HRQL.

RESULTS: Two-hundred and fifty patients with CLD and fifty normal subjects were enrolled into the study. Mean age and the numbers of low educated, unemployed, blue-collar career and poor health perception increased significantly from chronic hepatitis to Child's Classes A to B to C. Advanced stage of CLD was related to deterioration of HRQL. Increasing age and female reduced physical health area. Low socioeconomic factors and financial burden affected multiple areas of HRQL. In overall, the positive impact of self-rating health perception on HRQL was consistently showed.

CONCLUSION: Advanced stages of chronic liver disease, old age, female sex, low socioeconomic status and financial burden are important factors reducing HRQL. Good health perception improves HRQL regardless of stages of liver disease.

Key words: Health-related quality of life; Cirrhosis; Chronic hepatitis; Short-form 36; Chronic liver disease questionnaire

Sobhonslidsuk A, Silpakit C, Kongsakon R, Satitpornkul P, Sripetch C, Khanthavit A. Factors influencing health-related quality of life in chronic liver disease. *World J Gastroenterol* 2006; 12(48): 7786-7791

http://www.wjgnet.com/1007-9327/12/7786.asp

INTRODUCTION

In 1947, the World Health Organization expanded the definition of health to include in addition to the absence of disease, a complete state of physical, mental and social well-being[1]. Health-related quality of life (HRQL) emerges as a tool for measuring outcome from the patient's viewpoint, incorporating social, psychological, physiological and physical functioning[1,2]. Combined using generic and disease-specific instruments can provide more accurate assessment of both the global aspects and the specific features of HRQL of a specific condition[1]. The assessment of HRQL has been done in gastrointestinal diseases and chronic liver disease (CLD)[3-7]. It has been reported that the presence of CLD reduce HRQL and the deterioration of HRQL is apparent while the severity of disease increases[8-13]. Furthermore, demographic factors such as age and gender, alcohol, co-morbid illness, disease awareness and psychological status can affect HRQL in CLD[8-15]. However, a recent study showed that active psychiatric illness and medical co-morbidities, but not severity of liver disease, were determinants of HRQL reduction[16]. Previous researches of HRQL in normal and chronic medical conditions showed that socioeconomic and demographic factors can influence HRQL[17-20]. The contribution of socioeconomic factors and health perception to HRQL was not known in CLD. Self-rating patient health perception is one of the strongest predictors of mortality[21]. HRQL in CLD may be improved by changing patient health perception if there is a relationship between health perception and HRQL. The impact of marital status on HRQL is our interest because its significance had never been studied in CLD[8-13]. Our assumption was that married couple would have more psychosocial and emotional support than single, unmarried

or divorced people. An earlier study revealed that HRQL in Thai patients with CLD was lower than that of normal subjects similar to the reports from Western countries[22]. We aimed to investigate variables that truly affected HRQL, such as disease severity, etiology of liver disease, demographic and socioeconomic factors, and patient health perception in Thai patients with CLD.

MATERIALS AND METHODS

Study design and population

A cross-sectional study was carried out at the Gastroenterological Clinic between 1st January 2004 and 30th June 2004. Eligible patients with CLD, age 15-80 years, both men and women, were enrolled consecutively into the study. Exclusion criteria were the concomitant presence of hepatic encephalopathy, active medical co-morbidity, malignancy, current or previous treatment of antiviral agents and those who refused to participate with the study. CLD were classified into chronic hepatitis and cirrhosis. Chronic hepatitis was defined by the elevation of serum transaminase higher than 1.5 times of upper normal limit for 6 mo. The diagnosis of cirrhosis was confirmed from clinical finding, biochemical test, ultrasound or liver histology[23]. The staging of cirrhosis was graded according to Child-Pugh classification: Child's classes A, B and C[24]. Causes of CLD were divided into viral hepatitis, alcohol, viral hepatitis combining with alcohol, non-alcoholic fatty liver disease and miscellaneous causes. Alcohol was the etiology of CLD if daily alcohol drinking was greater than 40 g for at least 10 years. The cause of CLD was viral hepatitis B if hepatitis B surface antigen (Abbott Laboratories, North Chicago, IL) was positive, or viral hepatitis C if antibody to hepatitis C virus (anti-HCV) (Abbott Laboratories, North Chicago, IL) was positive. Data were collected from patient inquiry and medical records. Normal subjects who did not have medical illness were invited into the study. The study protocol was approved by the Hospital Ethical Committee and it was carried out according to the Helsinki Declaration Guidelines[25]. Written informed consent was obtained prior to the study.

Data collection

HRQL instruments (dependent variables): The study patients were asked to self-administer the short-form 36 (SF-36) heath survey and chronic liver disease questionnaire (CLDQ), and the answered questionnaires were checked for completeness by a research assistant who also helped interviewing illiterate patients for the questionnaires. The SF-36 consists of 36 items which are categorized into 8 domains of physical functioning, role-physical, bodily pain, general health, vitality, social functioning, role-emotional and mental health ranging from 0 to 100 with higher scores reflecting better perception of health. Physical functioning, role-physical, bodily pain and general health represent physical health scale, whereas vitality, social functioning, role-emotional and mental health define mental health scale. The domain scores were calculated according to the standard reference[26].

There are 29 items in the CLDQ summarized into 6 domains of abdominal symptoms, fatigue, systemic symptoms, activity, emotional function and worry. Each item consists of 7 linkert scales. Domain score is calculated from the average score of all items of that domain[7]**.** Both questionnaires were formally translated from the original versions and the validation of the questionnaires was reported elsewhere[22,27].

Definition of study variables (independent variables): Clinical, demographic and socioeconomic data were collected from each subject. Marital status was dichotomized into single and paired. Single was extended to include unmarried person, divorced or deceased couple. Socioeconomic status was assessed by using the level of education: lower than bachelor's degree and equal to or higher than bachelor's degree; presence and types of career: unemployed, blue-collar and white-collar; presence or absence of financial burden. Subjects were asked to rate their health as "very good", "good", "fair", "poor" or "very poor". Good health perception included "very good", "good" and "fair". Poor health perception consisted of "poor" and "very poor".

Statistical analysis

Data were entered into Excel spreadsheet (Microsoft Corporation) and analyzed using SPSS (version 11.5; SPSS, Inc., Chicago, IL) Categorical data are described as number and percentage [*n* (%)]. Continuous data were presented as mean ± SD and median (range). Statistical analysis of continuous data was performed with One-way Anova or non-parametric methods as appropriate. χ^2 test was used for analysis of discrete data, which give us the preliminary understanding of the association of the HRQL and studied variables. Stepwise multiple regression analysis was used to study the influence of independent variables on the CLDQ and SF-36 domains while controlling the effect of other variables. *P* value less than 0.05 was considered statistically significant

RESULTS

Characteristics of the study population

A total of 364 patients with CLD attended the Gastroenterology Clinic during the 6-mo period. Of these, 114 patients were ineligible for the study: 80 patients were either currently receiving or had received antiviral therapy; 17 patients had hepatocellular carcinoma; 13 patients had active co-morbid illness; two patients were having hepatic encephalopathy and two patients refused to participate in the study. Two-hundred and fifty subjects with CLD, and 50 normal subjects were enrolled into the study. Mean age (range) of the whole group was 48.1 (18-77) years. The number (%) of male to female ratio was 188:112 (62.7%:37.3%). The details of clinical, demographic and socioeconomic data are shown in Table 1. The majority of patients in both groups was male and had education lower than bachelor's degree. Although both groups reported financial problems in equal proportion, the socioeconomic status of CLD group was inferior to that of normal group, which was shown from the higher number of unemployed subjects and blue-collar typed career in the former group ($P < 0.01$). It is not surprising that poor health perception

Table 1 Baseline data of chronic liver disease and normal groups

Variable	Chronic liver disease	Normal group	*P*
n	250	50	
Age (Mean ± SD, yr)	49.1 ± 8.5	47.9 ± 12.0	0.65
Sex			
Male	160 (64.0%)	28 (56.0%)	0.33
Marital status[1]			
Single	71/238 (29.8%)	9/49 (18.4%)	0.07
Educational level[1]			
< Bachelor degree	165/237 (69.6%)	30/50 (60.0%)	0.12
Career[1]			
Unemployed	61/231 (26.4%)	3/46 (6.5%)	< 0.01
Blue-collar	37/231 (16.0%)	1/46 (2.2%)	
White-collar	133/231 (57.6%)	42/46 (91.3%)	
Financial burden[1]			
Present	87/238 (36.6%)	22/50 (44.0%)	0.20
Self-rating health perception[1]			
Poor health perception	61/238 (25.6%)	4/50 (8.0%)	< 0.01
Disease severity			
Chronic hepatitis	135/250 (54.0%)		
Child's class A cirrhosis	59/250 (23.6%)		
Child's class B cirrhosis	40/250 (16.0%)		
Child's class C cirrhosis	16/250 (6.4%)		
Causes of chronic liver disease			
Viral hepatitis B	99 (39.6%)		
Viral hepatitis C	48 (19.2%)		
Alcohol	43 (17.2%)		
Non-alcoholic fatty liver disease	27 (10.8%)		
Others	33 (13.2%)		

[1]Incomplete data.

Table 2 Variables by severity of the liver diseases

Variable	Normal	Chronic hepatitis	Child's class A	Child's class B	Child's class C	*P*
n	50	135	59	40	16	
Age (Mean ± SD, yr)	49.1 ± 8.5	43.5 ±12.2	51.7 ± 9.1	54.1 ± 10.2	54.6 + 8.0	< 0.01
Male	28 (56%)	88 (65.2%)	39 (66.1%)	22 (55%)	10 (62.5%)	0.73
Single[1]	9/49 (18.4%)	55/133 (41.4%)	7/53 (13.2%)	7/37 (18.9%)	3/15 (20.0%)	< 0.01
Low education[1]	30/50 (60%)	79/133 (59.4%)	39/53 (73.6%)	33/36 (91.7%)	14/15 (93.3%)	< 0.01
Unemployment[1]	2/50 (4.0%)	16/133 (12.0%)	11/53 (20.8%)	14/37 (37.8%)	3/15 (20.0%)	< 0.01
Blue-collar career[1]	1/46 (2.2%)	16/129 (12.4%)	8/51 (15.7%)	6/37 (16.2%)	7/15 (46.7%)	< 0.01
Financial burden[1]	22/50 (44.0%)	46/133 (34.6%)	22/53 (41.5%)	14/37 (37.8%)	5/15 (33.3%)	0.77
Good health perception[1]	46/50 (92.0%)	106/133 (79.7%)	38/53 (71.7%)	24/37 (64.9%)	9/15 (60.0%)	< 0.01

[1]Incomplete data.

was more frequent in the CLD than the normal group. In this study, there were only 16 (6.4%) patients with Child's class C cirrhosis, and viral hepatitis was the most common cause of CLD (58.8%), followed by chronic alcoholic (17.2%) and non-alcoholic fatty liver disease (10.8%).

Association of variables and disease severity

Similar to previous reports of any chronic liver diseases, male predominated in this study. The greatest number of single was found in chronic hepatitis group ($P < 0.01$). Mean age of this group was the lowest and age increased in advanced stages of CLD ($P < 0.01$). Low socioeconomic status, which was represented by lower education, unemployment and blue-collar typed career, increased in advanced stages of CLD. The reason of this finding is not known. Low socioeconomic status may keep the patients from appropriate treatment; hence the deterioration of liver disease is likely to happen. The proportion of good health perception decreased while the severity of CLD went up (Table 2).

The effect of disease severity on HRQL by univariate analysis

By univariate analysis, higher stages of CLD decreased HRQL in some domains of the SF-36, such as physical function, role-physical, general health and role-emotion ($P < 0.001$), and in all area of the CLDQ ($P < 0.03$). However, we could not make a conclusion that advanced

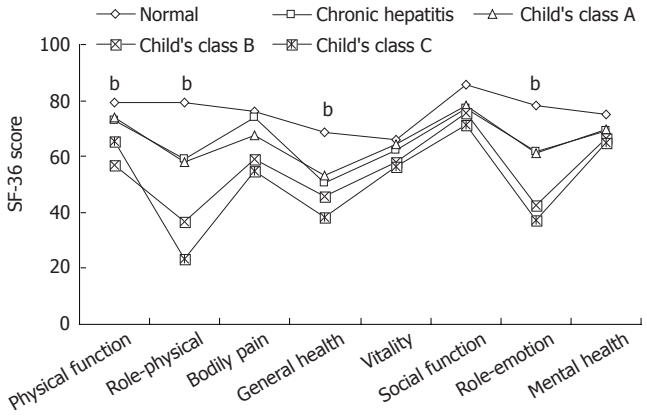

Figure 1 The domain scores of short-form 36 (SF-36) by disease severity. $^{b}P < 0.001$ *vs* normal group.

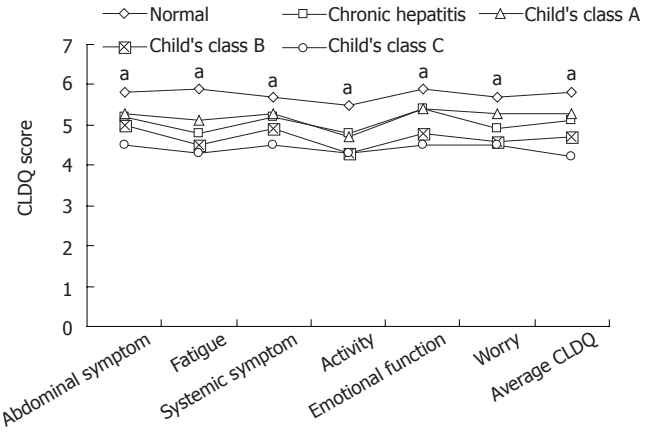

Figure 2 The domain scores of chronic liver disease questionnaire (CLDQ) by disease severity. $^{a}P < 0.03$ *vs* normal group.

Table 3 Variables affecting SF-36 domains[1]

Variable	Physical function	Role-physical	Bodily pain	General health	Vitality	Social function	Role-emotion	Mental health
Good health perception	13.7 (2.6)	36.4 (5.4)	21.6 (5.3)	26.2 (2.9)	17.2 (2.1)	15.8 (2.8)	23.1 (5.9)	18.0 (2.2)
Advanced stage	-3.1 (1.0)	-7.5 (2.2)		-4.0 (1.1)			-6.1 (2.4)	
Age (yr)	-0.4 (0.1)	-0.6 (0.2)	-0.8 (0.2)					
Female	-6.3 (2.2)							
Financial burden	-6.9 (2.2)	-15.8 (4.6)			-4.4 (1.8)		-17.0 (5.0)	-6.9 (1.9)
Low education					-4.7 (1.8)			
High level career							7.7 (3.0)	
F-statistic	18.5	26.2	18	55.6	32.2	31.7	13.3	42.8
R^2	0.26	0.28	0.12	0.29	0.26	0.1	0.17	0.24

[1]Only data with $P < 0.05$ are expressed as β-coefficient (SEM).

Table 4 Variables affecting CLDQ domains[1]

Variable	Abdominal symptoms	Fatigue	Systemic symptoms	Activity	Emotional function	Worry	Average CLDQ
Good health perception	1.1 (0.2)	0.9 (0.2)	0.9 (0.2)	0.7 (0.2)	0.9 (0.2)	1.0 (0.2)	1.0 (0.2)
Advanced stage	-0.2 (0.1)	-0.3 (0.1)	-0.2 (0.1)	-0.2 (0.1)	-0.3 (0.1)	-0.2 (0.1)	-0.3 (0.1)
Financial burden		-0.4 (0.1)		-0.3 (0.1)		-0.3 (0.2)	
F-statistic	29	22.2	24.5	19.2	36.3	16.1	25.2
R^2	0.18	0.2	0.15	0.18	0.21	0.15	0.22

[1]Only data with $P < 0.05$ are expressed as β-coefficient (SEM).

stages of CLD reduced the HRQL due to the presence of several confounding factors in advanced stages of CLD, such as old age, low socioeconomic status and poor health perception (Figures 1 and 2).

Influence of disease stage and variables on HRQL while controlling other variables

Multiple regression analysis of the association of HRQL domains and multiple variables such as stages of CLD, self-rating health perception, age, sex, financial burden, type of career, education level and viral hepatitis infection as a cause of CLD was performed. The advanced stages of CLD reduced all of the CLDQ domains, the majority of physical health scales of the SF-36 (physical functioning, role-physical and general health) and role-emotional domains. A one-year increase in age was associated with the reduction of 3 domains of physical heath scales of the SF-36 (physical functioning, role-physical and bodily pain), similar to the negative effect of female on physical functioning. While the presence of financial burden decreased multiple domains of the SF-36 and CLDQ, lower levels of education and career reduced predominantly the domains of mental health scales (vitality and role-emotion, respectively). Good health perception increased the SF-36 and CLDQ scores across the board. Viral hepatitis infection was not shown to affect any domains of HRQL (Tables 3 and 4).

DISCUSSION

Patients with CLD usually have HRQL lower than normal population, and the deterioration of HRQL appears while the severity of CLD increases[8-13]. This study focus not only on liver disease factors but also on other variables, such as age, sex, family support, socioeconomic status (education level, employment and career type), financial burden and self-rating health perception. Multiple regression analysis was performed to confirm the effect of variables on HRQL while controlling the influence of other variables. Advanced stages of CLD reduced all domains of the CLDQ, and the physical function, role-physical, general health and role-emotion domains of the SF-36. The effect of viral hepatitis infection as causes of CLD on HRQL reported from several studies is still inconclusive[15,28]. Recent systematic review revealed that the patients with HCV infection scored lower than the controls across all domains of the SF-36[27]. In our study, we could not find the impact of viral hepatitis infection, especially viral hepatitis C, on HRQL. However, the total cases of HCV infection in the study were quite low. There were only 48 (19.2%) patients with HCV infection distributing in three stages of cirrhosis and chronic hepatitis. In general, the elderly is associated with less favorable appraisal of personal health due to their health concerns, pessimistic health appraisals, social isolation and unemployment[29]. A previous study in CLD revealed that old age had a negative impact on HRQL[11]. Nevertheless, another study reported that cirrhotic patients with younger age had a more impairment in HRQL than the elder[9]. While important factors were controlled, a one-year increase in age reduced the scores of physical function, role-physical, and bodily pain from 0.4 to 0.8. In general, females have more health concerns and are more treatment-seeker than male. One study in CLD reported the minor effect of gender on HRQL in CLD[11]. We found that female gender yielded negative influence on physical functioning. Surprisingly, the marital status did not affect HRQL. This finding may be explained by the close-knit type of Thai society, so CLD patients could get psychosocial support from other family members even when they are single or divorced. Low socioeconomic status was shown to be important factor affecting HRQL in normal population and in patients with medical illnesses, such as prostate cancer, end-stage renal diseases and lung cancer[18-20,30]. Education level and career type were used as markers of socioeconomic status in this study because there is no standard categorization of socioeconomic status in Thailand. In general, education can help people cope their own problems. Low educated people are prone to have psychological problems and have false beliefs. People with lower socioeconomic status have more stress, more depression and interfamilial relationship problems in their life. As far as we know, there is only one study in chronic hepatitis C that reported the effect of education on HRQL[15]. We found that lower education level and type of career reduced vitality and role-emotion. The presence of financial burden can lower HRQL in several areas of the SF-36 and CLDQ. The impact of low socioeconomic status on HRQL supports the proposed conceptual model of HRQL by Wilson IB and Cleary PD in 1995, which states that socioeconomic factors influence multiple domains of functional status[21]. The most important contribution showed from our study is that self-rating patient health perception can affect HRQL in CLD. In the conceptual model, health perception is included in the model together with other factors, such as biological and physiological variables, symptom status, functional status, characteristics of individual and environment[21]. We found that the proportion of good health perception declined while the severity of CLD increased. Good health perception was the only factor shown to be positively associated with the SF-36 and CLDQ domains unanimously. This finding supports the HRQL model that health perception is related to functional status, symptom status, biological and physiological variables. It is possible that HRQL in CLD can be improved by searching strategy to increase patient's health perception. There is some evidence showing that psychological and emotional support can improve patient health perception[31].

In this study, we showed that the important factors that reduced HRQL in CLD included not only advanced stages of CLD but also old age, female sex, low socioeconomic status, financial burden, as well as poor health perception in accordance with the conceptual model of HRQL. We conclude that while medical treatment is a key to improve patient condition and HRQL, additional treatment with psychosocial support to raise patient health perception may improve HRQL, perhaps even better.

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COMMENTS

Background

Health-related quality of life (HRQL) in chronic liver disease patients is lower than normal population. Factors relating to the reduction of HRQL are inconsistently reported. The study of factors affecting HRQL in chronic liver disease in Asians has never been carried out.

Research frontiers

The data of several variables, e.g. disease severity, etiologic factor, demographic and socioeconomic, and patient self-rating health perception were collected. Then, multiple regression analysis was used to identify the factors that independently affect HRQL in chronic liver disease.

Innovations and breakthroughs

The study demonstrated that advanced stages of chronic liver disease, old age and female sex reduced HRQL in Thai patients. Furthermore, socioeconomic factors which hardly receive attention in previous studies of HRQL in chronic liver disease can affect HRQL. Importantly, this is the first time that patient health perception is shown to be strongly associated with HRQL in chronic liver disease.

Applications

While the medical treatment is a key to improve patient condition and HRQL, complementary treatment with psychosocial support aimed to raise patient health perception may improve HRQL. This conclusion needs further study to confirm.

Terminology

HRQL is a concept which reflects the physical, social, and emotional attitudes and behaviors of an individual as they relate to their prior and current health state. HRQL assessment describes health status from patients' perspective and serves as a powerful tool to assess and explain disease outcomes.

Peer review

This study concerns over the understanding of readers for the demonstration of results from multiple regression analysis. The key point of the analysis is to show if the presence of individual relating factor affects HRQL in chronic liver disease. Overall the paper requires grammatical work.

S- Editor Wang GP **L- Editor** Kumar M **E- Editor** Ma WH

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World J Gastroenterol 2006 December 28; 12(48): 7792-7797
World Journal of Gastroenterology ISSN 1007-9327

CLINICAL RESEARCH

Disease-specific health-related quality of life and its determinants in liver cirrhosis patients in Lithuania

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Received: 2006-09-26 Accepted: 2006-12-06

Key words: Liver cirrhosis; Quality of life; Chronic liver disease questionnaire; Case and control patients

Sumskiene J, Sumskas L, Petrauskas D, Kupcinskas L. Disease-specific health-related quality of life and its determinants in liver cirrhosis patients in Lithuania. *World J Gastroenterol* 2006; 12(48): 7792-7797

http://www.wjgnet.com/1007-9327/12/7792.asp

Abstract

AIM: To evaluate disease-specific quality of life (QOL) in liver cirrhosis patients and to compare it with those of a healthy population. Also an important objective was to assess whether QOL in liver cirrhosis patients differs by age and gender, by type and severity of disease.

METHODS: The case group of 131 liver cirrhosis patients was selected. The control group of 262 was enrolled from a healthy population according to the scheme of case-control study. Clinical, demographic, laboratory data were collected. QOL was measured with a specific chronic liver disease questionnaire (CLDQ), which was translated and validated in Lithuanian. QOL scores were compared between groups by age, gender, type and severity of disease. Cronbach's alpha statistics calculation was used for evaluation of internal consistency reliability. Student's *t* test or ANOVA were used for evaluation hypothesis about probability equation.

RESULTS: QOL was significantly lower in liver cirrhosis patients than in healthy population (59.5 ± 18.3 *vs* 85.3 ± 12.3, $P < 0.001$). The significant QOL differences between case and control groups were observed in domains of worry and abdominal symptoms, the smaller differences-in emotional functions and systematic symptom domains. Significantly worse QOL was in observed patients with increased clinical severity of the disease measured by Child-Pugh class. Age, gender and etiology of disease had an insignificant effect on QOL in cirrhotic patients.

CONCLUSION: QOL was significantly impaired in all CLDQ domains in liver cirrhosis patients. Increase in severity of disease was the major factor associated with poorer QOL.

INTRODUCTION

Measurement of quality of life (QOL) becomes increasingly important in clinical patient management[1-3]. The World Health Organization has expended and codified health definitions to multidimensional adding mental and social well being[4]. This allowed us, in the last decades of the 20th century, to develop quality of life concepts and adopt different instruments for multidimensional evaluation of health[5-9].

The main reason why the rapid development of QOL measures in health care took place was the growing recognition of the importance of understanding the impact of healthcare interventions on the patients' every day life, rather than only treatment of their bodies[10]. Also physicians have always intended to find out and better understand how their patients feel. This is particularly important for patients with chronic, disabling or life threatening diseases, in persons, who live with minor expectation to be cured and with conditions that are likely to impact their physical and social well-being. In such patients it may be more relevant than length of life, because they are frequently more concerned about quality and disability than about longevity[11,12].

Health-related QOL is important in measuring the impact or burden of a chronic disease. Liver cirrhosis is an example of such a disease. Patients with chronic liver disease suffer from: fatigue, pruritus, loss of esteem, depression, and other complications of cirrhosis such as hepatic encephalopathy, ascites, spontaneous bacterial peritonitis and recurrent variceal hemorrhages[13,14]. Some of these conditions have obvious clinical manifestation and could be easily measured by the traditional clinical outcome measures (ascites, spontaneous bacterial peritonitis, varicel hemorrhages). Other important conditions (fatigue, loss of

esteem, inability to function or work, anxiety, depression, emotional problems) are poorly evaluated by the clinical measures. More evidence that measuring QOL provides a better measurement of these latter conditions is presented in the recent literature[15-17].

The general aim of this study was to evaluate QOL in patients with liver cirrhosis and to compare its features with the control group of persons, selected from the population sample. We aimed also to look for associations between QOL scores and demographical characteristics, type of cirrhosis, severity of disease.

MATERIALS AND METHODS

Patients

The study was conducted during a one-year period in 2001-2002. In the first stage of the investigation the case group was selected (131 patients with cirrhosis). The control group (262 persons) was selected from randomly selected population according to the scheme of case-control epidemiological study. The group of cases was composed from patients with liver cirrhosis of different etiology. These patients have been admitted, diagnosed and treated at the Clinic of Gastroenterology, Hospital of Kaunas University of Medicine. The diagnosis was verified according to the data of anamnesis, clinical, biochemical and instrumental examinations and the results of percutaneous or transjugular liver biopsy data. Only the persons without hepatic encephalopathy, according to psychomotor tests, were included into the study.

The control group was randomly selected from the list of Kaunas county population. Pair matching method was applied in order to select the control group. The control persons were selected according to gender, age and the education background. Two controls were selected for one case person.

Methods

The routine clinical examination was carried out for the patients with liver cirrhosis: clinical and biochemical blood sample analysis, ultrasound investigation, esophagogastroscopy, percutaneous or transjugular liver biopsy. These persons were classified according to the etiology of disease. Clinical and biochemical analysis, evaluation of failure of liver function, also commonly manifested complications of cirrhosis were recorded and analyzed. Severity of liver cirrhosis was evaluated according to Child-Pugh score[18].

Clinical and epidemiological investigation methods were used. Investigation data of case groups were registered in the Registration Form for Clinical Data. General data about the cases and controls were collected in the General Questionnaire Form. QOL questionnaires were administered for both respondents of case and control groups.

The chronic liver disease questionnaire (CLDQ) was applied as the instrument for measuring QOL. This QOL investigation instrument was developed at the Department of Gastroenterology, The Cleveland Clinic Foundation by Younossi *et al* in 1999 as the disease specific instrument for evaluating QOL of patients with chronic liver disease[19]. Approval of the authors was received to use this instrument in our study. CLDQ covers 29 items and is designed to measure the six domains of QOL: abdominal symptoms (AB), fatigue (FA), systemic symptoms (SY), activity (AC), emotional functions (EM) and worry (WO). CLDQ has been translated to Lithuanian and passed validation procedures before this study. Evaluation of reliability and validity was carried out. Cronbach's alpha (measure of internal consistency) of overall scores was 0.93, which was above the acceptable level of 0.70. Approval from Biomedical Ethics Committee was obtained and participants signed a written consent prior filing the questionnaires.

SPSS 10.0 for Windows was used for research analysis. Cronbach's alpha statistics calculation was applied for evaluation of internal consistency reliability in QOL questionnaire. Standard means for QOL scores with a 95% confidence interval were calculated. For evaluation of continuous variables the statistical mean (m) and standard deviation (SD) were computed. Student's *t* test or ANOVA were used for proving hypothesis about probability equation. Mann-Whitney or Kruskall-Wallis tests were used for comparison two or more independent variable groups. $P < 0.05$ was considered significant in two-tailed tests.

Table 1 Clinical and demographical data of liver cirrhosis patients (n = 131)

Variable	*n* (%)
Age (yr)	
< 40	23 (17.6)
40-50	34 (26.0)
51-60	33 (25.1)
> 60	41 (31.3)
Gender	
Male	68 (51.9)
Female	63 (48.1)
Etiology of disease	
Viral B and/or C cirrhosis	53 (40.5)
Alcoholic cirrhosis	50 (38.2)
Cholestatic cirrhosis	11 (8.3)
Other cirrhosis	17 (13.0)
Child-Pugh class	
Class A	32 (24.6)
Class B	72 (54.6)
Class C	27 (20.8)

RESULTS

At the baseline survey 131 patients with liver cirrhosis of different etiology were examined. Table 1 summarizes their demographic and clinical characteristics.

QOL in liver cirrhosis patients and in the control group

For the researches and clinicians it is important to know, which particular domains of QOL are most affected by liver cirrhosis. Figure 1 presents the distribution of mean scores of six QOL domains measured by CLDQ

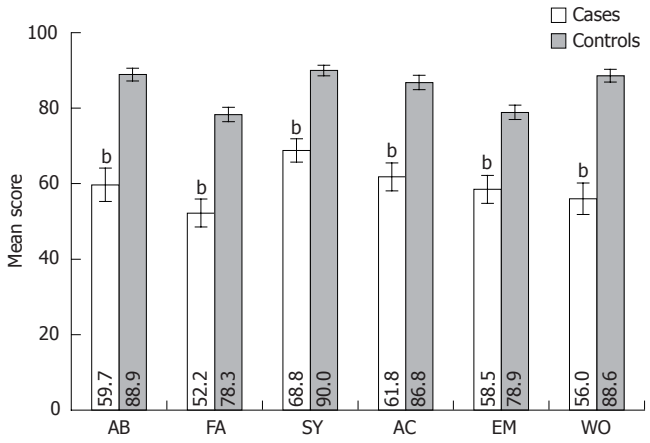

Figure 1 Chronic liver disease questionnaire (CLDQ) scale score differences in six quality of life (QOL) domains in case and control groups. Error bars indicate 95% confidence interval. AB: Abdominal symptoms; FA: Fatigue; SY: Systemic symptoms; AC: Activity; EM: Emotional function; WO: Worry. NS: Not significant. $^{b}P < 0.001$ *vs* control group.

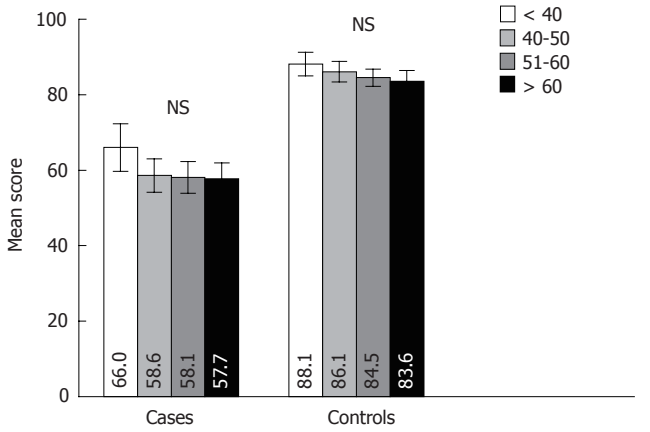

Figure 2 Chronic liver disease questionnaire (CLDQ) scale score comparison between the age groups in case and control groups. Error bars indicate 95% confidence interval. NS: Not significant.

questionnaire in cases and controls. It was established that in all six domains QOL mean score (SD) were lower in cirrhosis patients than in control group ($P < 0.001$). The most significant difference in QOL was observed in the domain of worry (56.0 ± 24.2 *vs* 88.6 ± 14.4, $P < 0.001$) and in abdominal symptoms (59.7 ± 25.8 *vs* 88.9 ± 14.2, $P < 0.001$). Smaller deterioration of QOL was established in the domain of emotional function (58.5 ± 20.9 *vs* 78.9 ± 16.1, $P < 0.001$) and in the systemic symptoms domain (68.8 ± 18.1 *vs* 90.0 ± 10.9, $P < 0.001$). The overall CLDQ score for patients with liver cirrhosis also was lower than in persons with no cirrhosis (59.5 ± 18.3 *vs* 85.3 ± 12.3, $P < 0.001$).

Age and QOL

The answers of respondents were analyzed in the four age groups. The distribution of patients by age groups was following: age group < 40 years-23 (17.6%), 40-50-year-old group-34 (26.0%), 51-60-year-old group-33 (25.1%) and > 60-years old group-41 (31.3%). The age structure

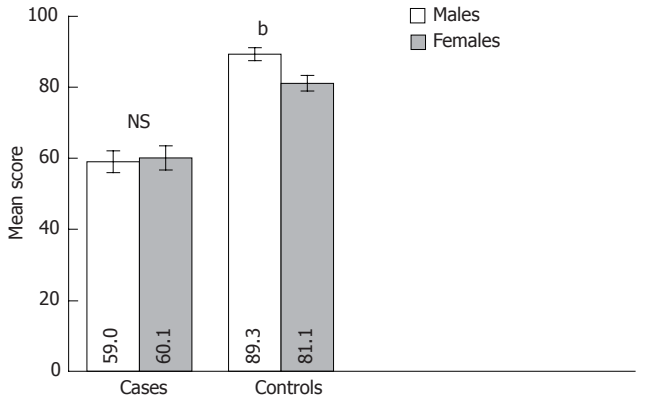

Figure 3 Chronic liver disease questionnaire (CLDQ) scale score comparison between the genders in case and control groups. Error bars indicate 95% confidence interval. NS: Not significant. $^{b}P < 0.001$ *vs* females in control group.

of the control group was the same according the design of study. Figure 2 presents data on distribution of QOL by age among the respondents in case and control groups. It is evident from this illustration that general QOL was decreasing only insignificantly during period of aging in both case and control groups.

Gender and QOL

The samples of cases and controls were composed of 51.9% of males and 48.1% of females. We have analyzed the effects of gender on QOL. Figure 3 illustrates how gender relates with QOL in both compared groups. It was proved that QOL is higher in control healthy males than in females ($P < 0.001$). However, no significant difference was established in QOL between genders in liver cirrhosis patients.

Type of liver cirrhosis and QOL

Comparison of QOL in patients with different types of liver cirrhosis was carried out in four groups of patients: alcoholic liver cirrhosis, viral liver cirrhosis, cholestatic liver cirrhosis (primary biliary cirrhosis and primary sclerosing cholangitis), and other forms of liver cirrhosis. It was established by our analysis that in all six CLDQ domains QOL was at the similar score level in all four groups of patients analyzed (Figure 4).

Severity of the disease according the Child-Pugh scale and QOL

We have analyzed and compared QOL in patients attributed to A, B and C liver cirrhosis severity classes according to Child-Pugh classification (Figure 5). The CLDQ showed significant worsening of QOL in parallel with increase of the clinical severity of disease measured by Child-Pugh scale (QOL mean score [SD] in A and C classes were respectively 65.9 ± 18.6 and 52.6 ± 17.0, $P < 0.01$).

DISCUSSION

Despite the fact that QOL investigations cover more and more diseases and population groups, its application in hepatology is still very scarce. Many recent publications

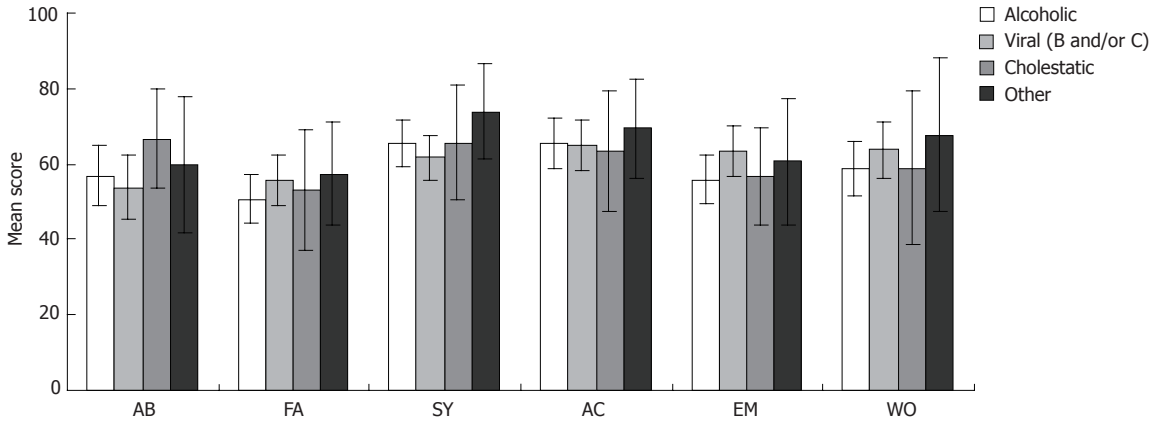

Figure 4 Chronic liver disease questionnaire (CLDQ) scale score comparison in six quality of life (QOL) domains by the etiology in liver cirrhosis patients. Error bars indicate 95% confidence interval. AB: Abdominal symptoms; FA: Fatigue; SY: Systemic symptoms; AC: Activity; EM: Emotional function; WO: Worry.

still agree that very limited information is available on the impact of liver cirrhosis on QOL and continue investigating different QOL instruments.

This investigation is important as an example where both population based and clinical approaches in research were applied by selecting patients (from hospital) and controls (from population). Also our study was new for our country and for other countries of Central and Eastern Europe because QOL of liver cirrhosis patients was not investigated before in this European region.

By permission of CLDQ authors the specific questionnaire to measure health related QOL in patients with chronic liver disease was translated, adopted and validated in Lithuania. CLDQ previously have been adapted and validated for German speaking countries and also recently was translated into Farsi, Thais[20-22].

In our study QOL of patients with cirrhosis was compared with a randomly selected population group, because CLDQ is not designed exclusively as the liver disease specific instrument and allows us to answer all the questions for healthy persons also. The extent of impaired QOL of cirrhosis patients differed in the various domains. The most significant decrease in QOL was observed in the domain of worry and in abdominal symptoms. Smaller deterioration of QOL was established in the domain of emotional function and in the systemic symptoms domain.

The author of CLDQ, Younossi, also has established negative impact of different chronic liver disease (primary biliary cirrhosis, viral hepatitis B and C, primary sclerosing cholangitis, hepatocellular disease) for QOL and has established that deterioration of QOL is similar as in patients with chronic obstructive pulmonary disease or congestive heart failure[23,24]. Similar findings, which indicate significantly lower QOL in liver cirrhosis patients, were presented by Italian survey, where short form-36 (SF-36) and Nottingham Health Profile (NHP) questionnaires were used and by Croatian authors, who used SF-36 instrument[25,26].

We have calculated how much QOL is affected in cirrhosis patients in relation to age and gender. It is evident from population-based studies that QOL decreases with age in normal population[2,3,25]. However, in our study QOL

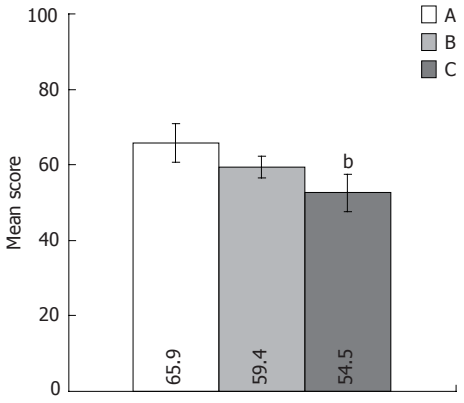

Figure 5 Chronic liver disease questionnaire (CLDQ) scale score comparison according the disease severity in the case group. Error bars indicate 95% confidence interval. A, B and C represent Child-Pugh class A, B and C, respectively. $^{b}P < 0.01$ *vs* A Child-Pugh class.

both in control and cirrhosis patients groups showed only small and not significant impairment with age ($P > 0.05$). In an Italian study, where SF-36 was used, a minimum deviation from population norms was established in the oldest group[25]. This could be explained, that in our cirrhosis group proportion of patients with more severe disease (higher Child-Pugh class) was higher than in the Italian study. On the other hand "normative" populations in these two studies could have different age structure and levels of QOL.

Our study has demonstrated that QOL is higher in males from random population than in females. However, gender did not show any effect on QOL of liver cirrhosis patients. Majority or researchers, who analyzed QOL data of cirrhosis patients also, have stated that QOL is not determined by gender[27-29].

We have classified cirrhosis patients according to the disease etiology into four groups: viral, alcoholic, cholestatic and other origin. It was established by our analysis that QOL do not differ significantly in all four groups. This fact could indicate that etiology has minor impact for QOL. Our results are in accordance with other surveys, which established the similar patterns[23,24].

However, literature indicates on significant effects of disease severity and worsening of QOL across the disease stages[23-25,30]. In our study chronic liver disease stages were classified into three groups (A, B and C) according to Child-Pugh score. We also established that higher severity of disease (higher Child-Pugh class) was associated with a lower CLDQ score.

Lithuania is a relatively small country and the number of liver cirrhosis patients that could be accessed at the university hospital during one year, is not big. We made an attempt to diminish this possible limitation by selecting two-fold larger control group from randomly selected "healthy" population and by matching case and controls. Groups of patients with liver cirrhosis represents clinical group of cases, which can not be considered as completely representing the whole population of patents with liver cirrhosis in the country. It is evident that in primary stages of disease the patients with liver cirrhosis have less probability to be referred to the hospital. Also severe patients with Child-Pugh class C could have encephalopathy and mental disorders-these were excluded from the study. On the other hand we should take into account the possibility of selection bias in the "healthy" group of controls-non-respondents, who are completely healthy, tend to refuse to fill in questionnaires. This could result in selection of the control group with lower QOL. These circumstances allow extrapolating the research inference for the whole population of cirrhosis patients with caution.

In summary, our data obtained by this survey have shown that general QOL and QOL in all health domains were lower in patients with cirrhosis than in controls selected from the normal population. The most significant QOL differences between case and control groups were observed in domains of worry and abdominal symptoms, the smaller differences-in domains of emotional functions and systematic symptoms. Disease severity (higher Child-Pugh class) was associated with lower Chronic Liver Disease Questionnaire score. Etiological type of liver cirrhosis had minor and insignificant effect on QOL.

ACKNOWLEDGMENTS

The authors would like to thank the administration of Hospital of Kaunas University of Medicine (head: Professor J Pundzius) for organizational support and input in providing facilities to conduct the study. Research Laboratory for Population-Based Studies (head: Professor S Domarkiene) of Institute for Cardiology of Kaunas University of Medicine has provided possibility to access random samples of normal population.

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S- Editor Wang GP **L- Editor** Alpini GD **E- Editor** Bai SH

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World J Gastroenterol 2006 December 28; 12(48): 7798-7804
World Journal of Gastroenterology ISSN 1007-9327

CLINICAL RESEARCH

Clinical research on navel application of *Shehuang Paste* combined with Chinese herbal colon dialysis in treatment of refractory cirrhotic ascites complicated with azotemia

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Received: 2006-08-10 Accepted: 2006-11-22

Abstract

AIM: To explore the efficacy and mechanism of a novel therapeutic method of traditional Chinese medicine in patients with refractory cirrhotic ascites complicated with azotemia.

METHODS: Seventy-five cases of refractory cirrhotic ascites complicated with azotemia were randomly divided into 3 groups: comprehensive treatment (n = 29), simple treatment (n = 24), and control (n = 22). The basic treatment methods were the same in all groups, including liver protecting medicines, diuretics and supportive drugs. The control group underwent only the basic treatment. *Shehuang Paste* (SHP) was applied to the navels of the two treatment groups once a day for 30 d. Colon dialysis with Chinese herbs was administered to the comprehensive treatment group once every two days. Before and after treatment, we measured abdominal circumference, BUN, Cr, serum Na^+, urine Na^+/K^+, liver function, endotoxin content, NO, and ET-1. Color Doppler ultrasonography was conducted to measure the portal vein blood flow.

RESULTS: The total effective rate for ascites was 72.4% in the comprehensive treatment group, 45.8% in the simple treatment, contrasting with 18.2% in the controls. Between the two treatment groups and the controls, there were significant differences in the effective rates ($P < 0.01$, and $P < 0.05$). There was also a significant difference ($P < 0.05$) between the two treatment groups. Measurements of Cr and BUN showed higher values for the treatment groups, with the comprehensive better than the simple group ($P < 0.05$). Sera Na, urine Na/K were different, $P < 0.01$ between pre- and post-treatment in the comprehensive group, and $P < 0.05$ in the simple group. The treatment groups' endotoxin content was also significantly reduced ($P < 0.01$, and $P < 0.05$), with the comprehensive group better than the simple group ($P < 0.05$). Portal vein blood flow and NO content significantly reduced ($P < 0.05$), as did ET-1 content ($P < 0.01$). There were no significant changes in the control group ($P > 0.05$). The comprehensive treatment group's pre- and post-treatment portal vein and splenic vein blood flows showed a positive correlation to NO, ET-1 and endotoxin contents.

CONCLUSION: When treating refractory cirrhotic ascites complicated with azotemia, *Shehuang Paste* combined with Chinese herbal dialysis is better than Shehuang Paste alone for ascites resolution, azotemia, and endotoxin elimination. However, both methods on their own were also effective for reducing portal and splenic vein blood flow, and lowering the contents of NO, ET-1 in the two treatment groups.

Key words: Cirrhotic; Refractory ascites; Azotemia; Vasoactive substance

Tong GD, Zhou DQ, He JS, Zhang L, Chen ZF, Xiao CL, Peng LS. Clinical research on navel application of *Shehuang Paste* combined with Chinese herbal colon dialysis in treatment of refractory cirrhotic ascites complicated with azotemia. *World J Gastroenterol* 2006; 12(48): 7798-7804

http://www.wjgnet.com/1007-9327/12/7798.asp

INTRODUCTION

Cirrhotic ascites is one of a triad related to portal hypertension, which is not difficult to be cured if patients receive early treatment. Only about 10% of patients proceed to develop 'refractory ascites'. The International Ascites Club[1] definition of refractory cirrhotic ascites is that ascites is not obviously reduced after treatment (mainly by diuretics administration) or that early ascites recurrence can not be prevented by medicines after discharging liquid administration, which was defined by the Chinese experts based on the simple fact that patients' abundant ascites

lasts more than 3 mo[2]. Approximately 20% of the patients will go on to develop hepatorenal syndrome (HRS) within 1 year[3]. Once HRS develops, the mortality rate is almost 100%. In their development, hepatopathy and nephropathy interact with each other. Hepatocirrhosis and fluid and sodium retention, easily complicate any dysfunction of the renal blood stream. If treated with diuretics, azotemia may be induced. However, anuria induced by renal dysfunction can aggravate ascites. Thus it is very important that clinical research should be aimed to prevent, to treat early or to stop the development of hepatorenal syndrome[4].

The pathogenesis of refractory cirrhotic ascites is not well understood. There are many theories, but portal hypertension is generally accepted as the basis of ascetic formation. Neural-tumour reaction is induced by portal hypertension, and an endogenesic vessel activity abnormality in which endotoxin, NO and ET-1 play important roles. These factors lead to hyperdynamic circulation and an effective blood capacity deficiency. Renal blood stream kinetic and functional abnormalities are important in the pathogenesis[5].

The major medicinal treatments are an abundant discharge of liquid, supplemented by a large volume of protein or self-liquid retransfusion. However, it cannot sustain for a long time and a circulatory functional disorder can easily happen after massive liquid discharging. To date, a medicine to completely reverse low perfusion of nephridium has not been determined[6]. Thus, it is difficult to treat refractory cirrhotic ascites by the normal methods of internal medicine.

There are reports of herbs being applied to the navel for the treatment of cirrhotic ascites[7], indicating that the application of herbs to the navel for the treatment of cirrhotic ascites is possible. Such treatment can act fast, significantly reduce ascites, improve symptoms while it neither injures patient's right Qi ("life energy" according to the principle of traditional Chinese medicine) nor disturbs the water-electrolyte balance. But there are few reports about the application of herbs to the navel for the treatment of refractory cirrhotic ascites. Chinese herbal colon dialysis has for many years been applied for treating renal failure. The mechanism for this treatment is that the human colon has functions of absorption and secretion. The mucosa of the colon, a semi-dialytic membrane, can selectively absorb or secrete, using the ion grads in dialytic water, discharge poisonous metabolic productions as endotoxins, carbamide, ammonia, and absorb substances useful for human life from herbs. *Da huang* is the herb usually used. Chen HL[8] added *Fu zi, chuan xiong, Hua Q*[9] conjugated *Fu zi, Shui zhi,* and *Sheng mu li,* Chen HC[10] *Jin yin hua*, *Huai hua*, *Pu gong ying*, and *Duan mu li*. These treatments are mostly used to treat chronic renal failure or hemorrhagic fevers of nephritic syndrome complicated with acute renal failure. However, we have found no reports about these treatments being used for cirrhotic ascites complicated with azotemia.

This study used SHP applied to the navel in combination with Chinese herbal colon dialysis. It aimed to observe the clinical effect on 'refractory cirrhotic ascites', measure the levels of endotoxins, NO, ET-1 and portal vein blood flow. We sought to prevent or postpone the process of hepatorenal syndrome during azotemia.

MATERIALS AND METHODS

Clinical material

All 75 patients enrolled with refractory cirrhotic ascites were inpatients hospitalized in the authors' hospital from July 2002 to June 2005. They were randomly assigned to 3 groups by SAS software: 29 cases to the comprehensive treatment group; 23 male, 6 female; average age 43 ± 10 years; and average disease duration 6.1 ± 3.0 mo. There were 19 cases of hepatitis B-related cirrhosis, 2 of alcohol-related cirrhosis, 1 of blood fluke related cirrhosis, and 1 of autoimmune related cirrhosis; 24 to the simple treatment group, 17 male, 7 female, average age 43 ± 13 years, average disease duration 7.7 ± 3.5 mo. There were 20 cases of hepatitis B-related cirrhosis, 1 of alcohol-related cirrhosis, and 1 of blood fluke related cirrhosis. The level of ascites, Cr, BUN, age, duration of disease, and disease condition between the two treatment groups were comparable, without significant differences ($P > 0.05$).

Diagnostic criteria for refractory cirrhotic ascites

The diagnosis of refractory cirrhotic ascites was made with reference to guidelines contained in the literature. Inclusion criteria[1]: (1) Patients with a confirmed diagnosis of cirrhotic ascites; (2) Cases of refractory ascites, that is, previous treatments were either not satisfactory in dispelling ascites or there was an unavoidable early recurrence of ascites after discharge and the ascites had lasted more than 3 mo; (3) The patients' serum Na < 130 mmol/L, urine Na < 10 mmol/24 h, urine Na/K < 1, and glomerular filtrating rate (GRE) were below normal ranges. The level of Cr is 141-211 μmol/L[11].

Exclusion criteria

Patients were excluded if one of the following conditions was present: (1) Ascites originating from cirrhosis complicated with a malignant tumor; (2) Complications with acute digestive tract bleeding or hepatic encephalopathy; (3) Complications with primary diseases of cardiovascular, renal and hematopoietic systems, or with mental disease; (4) Anal stricture, internal or external hemorrhoids with active bleeding; and (5) Patients who did not meet the inclusion criteria, did not follow directions about taking medication, medical records were missed, or the effects and safety of the treatment were difficult to be determined.

Therapeutic methods

SHP Ingredients: Each piece of SHP consisted of snail flesh (approximately 30 g), *Moschus* 1 g , artificial *Calculus Bovis* 1 g, *Radix Euphorbiae Kansui* 10 g and *Bulbus Allii Fistulosi* 10 g.

Preparation: *Bulbus Allii Fistulosi* was pressed juice. *Radix Euphorbiae Kansui* was decocted with water twice, at each 2 h, and mixed with *Bulbus Allii Fistulosi* juice that had been filtrated. The mixture of the juice of the 2 herbs was concentrated to a dense paste with a relative density of 1.20. Moschus and artificial *Calculus Bovis* were extracted after being recirculated three times in 95% ethanol. Snail

flesh was minced, the dregs removed and then stewed. The extract of *Moschus* and artificial *Calculus Bovis*, the stewed snail product and the dense paste were mixed and then formed into a medicated patch of 3 cm × 3 cm in size (undertaken by the Pharmaceutical Department of Shenzhen Hospital, affiliated to Guangzhou University of TCM, batch No: 030113)[12]. The patch was placed into a bag and stored at 4℃.

Quality control: Quality control for the SHP was by detecting the content of Kotone musk and Bilirubin, the main ingredients of *Moschus* and artificial *Calculus Bovis*. *Moschus* (Batch No: 030912) and artificial *Calculus Bovis* (Batch No: 031221) were purchased from Shenzhen Branch of the Medicinal Material Company of China. The amount of medicinal paste on each patch was 1.2 g/100 cm^2. Bilirubin content in *Calculus Bovis* was above 41% (Bilirubin content is noted as being 35% in the 2005 edition of Pharmacopeia of China). Ketone musk in each piece of SHP was 2.73 mg/g[13] measured by high performance liquid chromatograph (Gillson, France). The method is reliable and stable.

Colon dialysis ingredients: The formula used for colon dialysis consisted of *Rheum Palmatum L*, Sophorage Japonica L, Lonicera Japonica Thunb, Taraxcum mongolicum, Herba-Mazz, and Ostrea telienwhanensis Crosse; 30 g of each.

Preparation: All ingredients in the colon dialysis formula were decocted together twice in a routine way. The 2 juices were mixed, filtrated to 200 mL, and stored at -70℃ until use. For use, the mixture was warmed to 38℃(carried out by the Preparation Room, Guangzhou University of Traditional Chinese Medicine affiliated Shenzhen Hospital, No. 030113).

Group treatment

The basic treatment was given to all three groups. Included in this were liver protecting medicines such as Wuzhi Jiaonang (mainly deoxyschizandrin, two tablets given each time, three times a day) and Silybin Meglumine (two tablets each time, three times a day); diuretics furosemide and antisterone at a ratio of 1:2 (80 mg: 160 mg); and supportive drugs such as 20% human albumin at a dosage of 100 mL per day. Ascites were discharged from 1000 mL to 1500 mL the first time and then from 2000 mL to 3000 mL each time for 3 times once every 2 d. Antibiotics (fortum 2.0 g/d) were administered to patients who complicated with peritonitis.

For the simple treatment group, the treatment method was the basic treatment plus SHP applied to the navel. For the comprehensive treatment group, the basic treatment plus SHP on the navel and the herbal colon dialysis. For the control group, only basic treatment .

Treatment procedures

Navel application: One piece of paste was compressed on the navel (Shenque acupoint) every day.

Colon dialysis: The following were the procedures carried out by patients; first, defecate and urinate. Incline on the right while lying down and prop the buttocks up by about 10 cm. Before dialysis, insert a SaveMedical double-cavity tube into the anus, insert the entering end about 50 cm into the inner anus and the exiting end would then be about 20 cm into the inner anus. Apply 1200 mL mannitol and 1200 mL dialytic fluid as an enema for 2 h. Clear the intestinal tube to maintain the high penetrability to intestinal mucosa. Later, insert 200 mL of the colon dialysis fluid for 30-60 min until an unforced defecation occurs. For the control group, a placebo of flour was applied to the navel, and the colon dialysis fluid was replaced with Sodium Chloride.

Period of treatment: One month made up one course of therapy. The following items were measured before treatment and at the end of treatment: abdominal circumference, BUN, Cr, serum Na^+, urine Na^+/K^+, liver function, endotoxin content, NO, and ET-1 nephritic syndrome. All items were then rechecked every month over a follow-up period of 3 mo.

Observational methods

General condition: Body weight, abdominal circumference, tongue and coating, pulse rate, volume of urine in 24 h, levels of urinary Na^+ and K^+, and Na^+/K^+ ratio in urine were measured every morning.

Blood Na^+ and liver function indexes: Blood Na^+, and indexes of liver functions including alanine transaminase (ALT), and aspartate aminotransferase (AST) were measured. Gamma glutamyl transferase (r-GT), total bilirubin (TB), albumin (ALB) and albumin/globulin ratio (A/G) and indexes of renal function, including serum Cr: liver function, serum Cr and BUN were checked using an Olympus 27000 (Japan) for routine automatic biochemical analysis once a week.

Levels of endotoxin, NO and ET-1: Levels of endotoxin, NO and ET-I were measured before and after treatment using 5 mL of fasting (8 h) cubital venous blood drawn in the morning. Plasma was separated and stored at -20℃. Plasma endotoxin was determined by limulus lysate chromogenic test, and NO by indirect colorimetry. The reagents used were provided by the Beijing Bangding Corporation of Biological Medical Science. ET-1 was determined by radio-immunoassay using a testing kit was provided by the East Asia Technological Institute, of the General Hospital of the People's Liberation Army. All tests were conducted by a trained technician using the same device and kits from the same batch.

Blood dynamics: Examination was conducted early in the morning on the day of blood collection. Patients were required to lie supinely and breathe calmly; a dual-function Color Doppler, Fynergy by GE, DIASONICS Corporation of America , with a 35 MHz frequency detector was used to measure the peak velocity of the blood flow (Vp) in the inner diameter(D) of the portal vein trunk and the splenic vein of the hilum . Examinations were carried out by a trained technician with the volume of the sampling as close as possible to that of the diameter of the blood vessel, and the angle between the sound beam and the blood flow as small as possible (less than 60º). All variables were measured twice and averaged. The volume of blood flow (Q) of portal and splenic veins were calculated by the formula [Vmean × $(D/2)^2\pi$ × 60] (D means diameter of blood vessel, Vmean means average velocity of blood flow)[14].

Table 1 Comparison of pre-and post-treatment for general efficacy on ascites *n* (%)

Treatment groups	*n*	Ascites grade Ⅰ resolution	Ascites grade Ⅱ resolution	Ascites grade Ⅲ resolution	No effect
Comprehensive	29	6 (20.7)	6 (20.7)	9 (31.0)	8 (27)
Simple	24	2 (8.3)	3 (12.5)	6 (25.0)	13 (54.2)
Control	22	0 (0.0)	1 (4.5)	3 (13.6)	18 (81.8)

Table 2 Changes of azotemia (mean ± SD)

Treatment groups	*n*		Cr (μmol/L)	BUN (mmol/L)
Comprehensive	29	Before	162.96 ± 11.73	10.54 ± 1.23
		After	113.82 ± 9.62[b,d]	6.48 ± 0.61[a,d]
Simple	24	Before	158.32 ± 17.21	10.41 ± 1.43
		After	123.54 ± 23.13[a,c]	7.92 ± 0.87[b]
Controls	22	Before	161.51 ± 11.55	9.09 ± 1.18
		After	143.41 ± 9.87	8.28 ± 0.38

[a]$P < 0.05$, [b]$P < 0.01$, pre-treatment *vs* post-treatment; [c]$P < 0.05$, [d]$P < 0.01$ *vs* control group.

Table 3 Pre- and post-treatment liver function changes (mean ± SD)

Groups	*n*		ALT (U/L)	AST (U/L)	γ-GT (U/L)	TB (U/L)	ALB (U/L)	A/G (U/L)
Comprehensive	29	Before	81.73 ± 55.31	96.92 ± 52.32	177.63 ± 22.24	59.89 ± 34.91	28.28 ± 4.19	0.82 ± 0.19
		After	53.22 ± 30.16[a]	83.56 ± 63.45	101.12 ± 44.11[a]	28.12 ± 10.23[a]	34.14 ± 3.95[a]	1.17 ± 0.74[a]
Simple	24	Before	73.67 ± 43.13	98.12 ± 62.26	170.03 ± 13.45	67.14 ± 41.39	28.38 ± 3.21	0.71 ± 0.18
		After	50.12 ± 20.43[a]	84.65 ± 60.50	88.20 ± 33.99[a]	35.22 ± 9.35[a]	35.47 ± 3.69[a]	1.15 ± 0.70[a]
Controls	22	Before	70.16 ± 37.24	102.12 ± 60.32	168.26 ± 118.04	68.81 ± 41.33	29.78 ± 3.39	0.72 ± 0.21
		After	51.02 ± 18.34[a]	87.65 ± 62.12	89.27 ± 35.19[a]	33.12 ± 9.08	34.78 ± 4.16[a]	1.11 ± 0.73

[a]$P < 0.05$, pre- and post-treatment comparison.

Efficacy evaluation standards

Using the "Standards for Efficacy Evaluation of TCM on Liver Cirrhosis Ascites" formulated at the Dalian Conference in 1993 by the special committee of Internal Heptatology, the China Association of Chinese Medicine (draft)[15], the efficacy on ascites was classified into 3 grades. Grade I: complete resolution of ascites, with no abdominal fluid found in ultrasound B examination, and condition stabilized for more than 3 mo; Grade Ⅱ: most of the ascites disappeared with only slight shifting dull sound upon percussion in a physical examination and ultrasound B shows little ascites; and Grade Ⅲ: ascites are somewhat diminished and the abdominal circumference at the level of the naval has decreased by more than 30 cm.

Statistic analysis

Variance analysis and χ^2 test were used to analyze all measurement and enumeration data, respectively. Linear correlation regression analysis was used for the relationship among PVQ and SVQ with levels of endotoxin, NO, and ET-1.

RESULTS

General efficacy for ascites in all groups

In most patients symptoms and physical signs improved to various degrees after treatment. Especially, abdominal distension, reduced urine, and abdominal circumference, lower limb swelling apparently improved, and body weight loss. However, fatigue, liver palm, and spider telangiectasia did not show any change.

In Table 1, the total effective rate on ascites in the treatment group is 72.4% (21/29), the simple treatment group 45.8% (11/24), and the control group 18.2% (4/22). There is no grade I resolution of ascites in the control group. By χ^2 test, there is a significant difference between the control and comprehensive treatment groups ($P < 0.01$), and the control and the simple treatment groups ($P < 0.05$). There is also difference between the two treatment groups ($P < 0.05$).

Changes of azotemia

Table 2 shows statistically significant differences between pre- and post-treatment changes of azotemia in the comprehensive treatment ($P < 0.01$) and simple treatment ($P < 0.05$) groups. The control group has no obvious change ($P > 0.05$). There are distinct differences between each treatment group and the controls ($P < 0.01$, $P < 0.05$). However, there is no difference between the two treatment groups ($P > 0.05$).

Changes of liver function, blood Na^+ and urine Na^+/K^+

Changes of liver function are shown in Table 3. Liver function ALT, TB, ALB, A/G are significant different between pre- and post- treatment stages in all three groups ($P < 0.05$), but there is no significant difference between the treatment and control groups. Changes of serum Na^+, and Na^+/K^+ are shown in Table 4. The serum Na^+, and Na^+/K^+ rates of the control group were increased after treatment, but there was no significant difference ($P > 0.05$). However, there are significant differences between before and after treatment in the comprehensive treatment ($P < 0.01$) and simple treatment ($P < 0.05$) groups. Though there is no statistical difference between the two treatment groups ($P > 0.05$).

Levels of endotoxin, NO, and ET

Endotoxin content decreased in the comprehensive

Table 4 Pre- and post-treatment serum Na^+ and urine Na^+/K^+ changes (mean ± SD)

Groups	*n*		Serum Na^+	Urine Na^+/K^+
Comprehensive	29	Before	126.32 ± 5.13	0.86 ± 0.23
		After	133.19 ± 3.21[b]	1.76 ± 0.65[b]
Simple	24	Before	127.27 ± 4.13	0.79 ± 0.26
		After	132.43 ± 3.34[a]	1.65 ± 0.85[a]
Controls	22	Before	126.16 ± 5.24	0.82 ± 0.32
		After	128.50 ± 7.29	1.25 ± 0.91

[a]$P < 0.05$, pre- and post-treatment comparison; [b]$P < 0.01$, pre- and post-treatment comparison.

Table 5 Pre- and Post-treatment changes of endotoxin, NO and ET-1 (mean ± SD)

Groups	*n*		Endotoxin (ng/L)	NO (μmol/L)	ET-1 (ng/L)
Comprehensive	29	Before	96.71 ± 28.82	15.82 ± 6.41	44.36 ± 7.14
		After	70.56 ± 27.34[b,d]	11.11 ± 6.02[a,c]	33.87 ± 8.95[b,c]
Simple	24	Before	95.27 ± 29.12	15.11 ± 6.99	45.59 ± 7.46
		After	80.69 ± 26.44[a,c]	11.63 ± 6.15[a,c]	36.67 ± 8.58[b,c]
Controls	22	Before	95.34 ± 30.22	15.65 ± 7.34	41.13 ± 8.25
		After	90.14 ± 32.38	14.23 ± 7.03	39.12 ± 8.46

$P < 0.05$, [a]Pre- and post- treatment comparison, [b]$P < 0.01$; [c]comparison between groups, $P < 0.05$, [d]$P < 0.01$.

Table 6 Pre- and post-treatment blood flow of three groups (mean ± SD)

Groups	*n*		PV (Portal vein)			SV (Splenic vein)		
			D (cm)	Vp (cm/s)	Q (mL/min)	D (cm)	Vp (cm/s)	Q (mL/min)
Comprehensive	29	Before	1.43 ± 0.18	14.15 ± 3.34	1274 ± 429	1.19 ± 0.22	15.27 ± 4.12	896.5+301.8
		After	1.21 ± 0.22[a,c]	15.27 ± 3.23	906.0 ± 316[a,c]	1.07 ± 0.18	15.13 ± 3.78	592.2 ± 201.8[a,c]
Simple	24	Before	1.42 ± 0.16	14.21 ± 3.06	1249 ± 416	1.20 ± 0.24	14.26 ± 4.10	896.5+301.8
		After	1.23 ± 0.32[a,c]	14.95 ± 3.40	896.0 ± 376[a,c]	1.06 ± 0.25	15.10 ± 3.80	609.2 ± 208.7[a,c]
Controls	22	Before	1.41 ± 0.26	14.20 ± 2.20	1238.2 ± 422.0	1.20 ± 0.22	14.84 ± 2.56	853.1 ± 321.2
		After	1.40 ± 0.22	14.01 ± 2.40	1247.0 ± 364.2	1.19 ± 0.23	14.96 ± 4.00	843.7 ± 341.0

[a]$P < 0.05$, comparison of pre- and post-treatment; [c]$P < 0.05$, *vs* control group.

treatment ($P < 0.01$) and simple treatment ($P < 0.05$) groups. NO ($P < 0.05$) and ET-1 ($P < 0.01$) also decreased after treatment. There was no significant difference between the two treatment groups ($P > 0.05$). Endotoxin, NO and ET-1 contents of the control group also showed no statistical difference ($P > 0.05$) (Table 5).

Comparison of blood flow in portal and splenic veins

For both treatment groups, the diameter (D value) and quantity of blood flow (Q value) of the portal and splenic veins decreased significantly after treatment ($P < 0.05$). In the control group, no great change was found after treatment ($P > 0.05$). Comparison of D and Q among the treatment and control groups after treatment found significant differences ($P < 0.05$). But there were no significant difference between the two treatment groups ($P > 0.05$). No significant changes were shown in Vp in all three groups before and after treatment ($P > 0.05$) (Table 6).

Pre- and post-treatment correlations between splenic and portal vein blood flows and endotoxin, NO and ET-1

As shown in Table 7, the PVQ and SVQ show a positive correlation to the pre- and post-treatment endotoxin levels in the 29 cases of the comprehensive treatment group with a pretreatment r (PVQ) = 0.67, r (SVQ)= 0.73 for $P < 0.01$; and post-treatment r (PVQ) = 0.75, r (SVQ) = 0.69 for $P < 0.01$. They have a positive correlation to the NO level with a pretreatment r (PVQ) = 0.68, r (SVQ) = 0.68 for a $P < 0.01$; and a post-treatment r (PVQ) = 0. 45, r (SVQ) = 0.51 for $P < 0.05$. They also have a positive correlation to ET with a pre-treatment r (PVQ) = 0.79, r (SVQ) = 0.78 for $P < 0.01$; and a post-treatment r (PVQ) = 0.73, r (SVQ) = 0.74 for $P < 0.01$.

Adverse reactions and follow-up

Five patients in the treatment groups showed papilla, reddening, swelling or itching skin where paste was applied to the naval area. The symptoms were endurable after treatment with dexamethasone acetate, ointment and there was no need to discontinue the paste application. No other kinds of allergic or adverse reactions were seen. Colon dialysis was implemented every two days and all 53 patients completed the treatment.

During the 3 mo follow-up, 7 cases control dropped out (3 cases effectively, 4 ineffectively treated). In 4 effectively treated patients, the ascites returned to the same level as pre-treatment; 2 ineffectively treated cases died because of HSR. In the treatment groups, 13 cases dropped out (8 effectively, 5 ineffectively treated). In 11 effectively treated patients, the ascites returned to the same level as pre-treatment; 20 cases remained in a stable condition; and in 9 cases the ascites increased but was were less than before treatment. No patient in the treatment groups was found to suffer from HSR.

DISCUSSION

For a long time, we have been using SHP applied to the navel area to treat refractory cirrhotic ascites, and have achieved good efficacy[16]. SHP is useful to treat refractory cirrhotic ascites; however, if complicated with azotemia the efficacy is not so good. Therefore, we have combined this with a herbal colon dialysis treatment; in accord with the theory of colon dialysis for treating uremia. The *Moschus* in the SHP aromatically opens orifices, unblocks network vessels and disperses stasis. Modern research shows that it dilates the blood vessels and has the same curative effect as

nitroglycerin[17]. The Snail ingredient clears heat, disinhibits fluids, and treats jaundice. The artificial *Calculus Bovis* clears the heart and disinhibits the gallbladder; likewise, modern research shows that it safeguards the liver and disinhibits the gallbladder[17]. Bulbous Allii Fistulosi frees Yang (the bright positive masculine principle in Chinese dualistic cosmology), unbinds toxins, and can guide medicinals to the affected site; likewise, modern research shows it has bacteriostatic capabilities[17]. *Radix Euphorbiae Kansui* drastically precipitates and expels water. The *Rheum Palmatum L* in the colon dialysis formula removes residues in the stomach intestine, reduces the reabsorbing of azotic, restrains decomposition of BUN and Cr, and has bacteriostatic capabilities[17]. *Lonicera Japonica Thunb, Taraxcum Mongolicum Herba-Mazz* have heat-clearing and detoxification effects; again, modern research shows they have broad-spectrum bacteriostatic capabilities[17]. Sophorage Japonica L clears heat and cools the blood; it can improve filtering capabilities of kidneys[17]. *Ostrea Teliennhanensis Crosse* retains Yin (the dark negative feminine principle in Chinese dualistic cosmology) and suppresses yang; it can also absorb toxic substances in the intestines. Both SPH and the colon dialysis formula dissipated blood stasis and disinhibited the gallbladder, clearing away heat and toxins, and eliminating fluid by purging.

In our 75 cases presented here, after the basic treatment that included liver pretecting, diuretics, human albumin, and antibiotics, liver function ALT, TB, ALB, A/G in all treatment and control groups had obvious improvements. There was no significant difference between each of the treatment and control groups. However applying herbs on the navel plus herbal colon dialysis was found to not improve the liver function compared with the basic treatment.

After the 3 groups underwent treatment, some improvements in some symptoms were seen. Taking the resolution of ascites as the major efficacy standard, the general rate of the effectiveness of the comprehensive treatment was 72.4%, the simple treatment group was 45.8% (11/24), while that of the control group was 18.2% (4/22). No patient reached Grade I for resolution of ascites in the control group. The effective rate of the comprehensive treatment group was higher than that of the simple treatment group ($P < 0.05$). From this we can infer that only using the basic treatment to treat refractory ascites cannot reach the therapeutic goal, putting herbs on the navel as a treatment has some effect on refractory ascites complicating with azotemia, but combining this with herbal colon dialysis can elevate the efficacy. The three-month follow-up here showed the lasting effects. From an assessment of the indexes having relationships with clinical azotemia, such as Cr, BUN, serum Na, and urine Na^+/K^+, there was no efficacy pre- and post-treatment in the control group. However, there was a significant difference between the simply treatment group and the comprehensive treatment group, which is better than using just SHP in addition to the basic treatment.

The main pathogenesis of refractory cirrhotic ascites is due to portal vein hypertension (PVH). Therefore, this study sought to show the way to lower PVH. PVH forms as a result of mechanical and functional factors.

Table 7 Pre- and post-treatment correlation between splenic and portal vein blood flows and endotoxin, NO and ET-1 (mean ± SD)

Indexes	*n*		*r*	
			PVQ	SVQ
Endotoxin	29	Before	0.67[b]	0.73[b]
		After	0.75[b]	0.69[b]
NO	29	Before	0.68[b]	0.68[b]
		After	0.45[a]	0.51[a]
ET-1	29	Before	0.79[b]	0.78[b]
		After	0.73[b]	0.74[b]

[a]$P < 0.05$, [b]$P < 0.01$.

First, patients present increased blood flow resistance and blood volume in the portal vein. Then they exhibit PVH. We observed portal vein trunk hemodynamic changes by color Doppler ultrasonography; showing that the portal vein blood flow was reduced (mainly by decreasing the diameter of the portal vein) in the two treatment groups after treatment; there was no difference between the two treatment groups. We highlighted that the improvements in PVH came mainly because of SHP. SHP possibly activates blood circulation to dissipate blood stasis, and has the effect of reducing platelet coagulation, improving circulation in the liver and decreasing portal vein resistance. Also, SHP can decrease vasoactive substances, slow down hypertension in the mesentery, and work toward decreasing PVH. Thus it appears a real way to reduce ascites.

PVH is also the initiating factor for the release of vasoactive substances. An increase of endotoxin during cirrhosis stimulates iNOS synthesis, which releases a significant quantity of NO[18]. The increased amount of NO released in the body leads to dilation of the peripheral vascular system, decreased arterial pressure and effective blood capacity. The decrease in arterial pressure then stimulates the sympathetic nervous system and activates the rennin-angiotonin-aldosterone system to alter the compensatory balancing of hemodynamics. As the illness continues, there is further compensation, even excessive compensation that can ultimately cause a decrease in the amount of blood circulating in the body, retention of both fluid and sodium, a redistribution of the renal blood flow, and the appearance of ascites, edema and azotemia[19]. The peripheral vascular dilation caused by NO inevitably stimulates the body to compensate by synthesizing and releasing large quantities of ET-1. The increase of ET-1 leads to the contraction of blood vessels in the liver and kidneys, aggravating them to ischemia and portal shunting causing increased endotoxins to enter into the circulatory system; thus initiating a vicious cycle[20]. A reason why refractory ascites is difficult to treat is that as it appears, the amounts of NO, ET-1, and endotoxin also gradually increase[21]. Our research results also show that ascites have a positive correlation to the amount of portal vein blood flow and vasoactive substances. In refractory ascites patients, NO, ET and endotoxin are at an elevated level, together with the ongoing ascites. With ascites eliminated after treatment, the levels of endotoxin, NO and ET-1 correspondingly decreased.

Refractory cirrhotic ascites easily reduces azotemia, which also has an early role in hepatorenal syndrome. For treatment, it is effective for ascites to improve intrahepatic circulation and decrease portal vein blood flow, but this is not sufficient for ascites associated with azotemia. Thus, along with the application of Shehuang Paste to the navel area, we combined a Chinese herbal colon dialysis. The crucial procedure in the method is that patients undergo dialysis 2 h daily using mannitol and peritoneal dialysis liquid, and then a colon-enema for 30 min with a Chinese herb decoction. The mechanism utilizes the high diosmosis of the colonic semi-permeable membrane caused by a high diosmosis dialysis liquid to absorb other liquids, and Chinese herbs to reduce the release of harmful and vasoactive substances, especially ammonia and endotoxin. All the cases here were refractory cirrhotic ascites complicated with azotemia. The results presented here demonstrated that applications of Shehuang Paste to the navel area in combination with Chinese herbal colon dialysis is superior to Shehuang Paste alone for eliminating ascites, lessening azotemia, and for reducing vasoactive substances such as endotoxin.

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S- Editor Wang J **L- Editor** Ma JY **E- Editor** Bi L

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World J Gastroenterol 2006 December 28; 12(48): 7805-7809
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

An algorithm for family screening for coeliac disease

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Received: 2006-08-07 Accepted: 2006-08-29

Abstract

AIM: To assess the level of undiagnosed coeliac disease (CD) in relatives of patients affected by the condition.

METHODS: We collected blood from 914 relatives of probands. We screened these individuals by ELISA for IgA and IgG tTG antibodies, confirming any positive IgA tTG results with an IgA EMA and looked for evidence of IgA deficiency in those who were IgG tTG positive alone, and performed IgG1 EMA in these individuals. We undertook HLA typing where positive screening was found, and this confirmed a strong prevalence of HLA-DQ2 in the coeliac population. Follow-up small intestinal biopsy was undertaken in cases with positive serological screening, wherever possible.

RESULTS: Use of this serological screening algorithm revealed a prevalence of undiagnosed CD in 5%-6% of first degree relatives of probands.

CONCLUSION: Our data suggests that first degree relatives of individuals with CD should be screened for this condition.

Key words: Coeliac disease; Screening; Endomysial antibody; Familial study; IgA deficiency; Prevalence; Tissue transglutaminase

Fraser JS, King AL, Ellis HJ, Moodie SJ, Bjarnason I, Swift J, Ciclitira PJ. An algorithm for family screening for coeliac disease. *World J Gastroenterol* 2006; 12(48): 7805-7809

http://www.wjgnet.com/1007-9327/12/7805.asp

INTRODUCTION

Coeliac disease (CD) is a disorder in which genetically predisposed individuals develop a small intestinal enteropathy on exposure to dietary gluten. The small bowel abnormalities are reversed on withdrawal of gluten from the diet. Recent population studies and serological testing of at-risk groups reveal a much higher prevalence of CD than previous studies. Whereas the previous prevalence was thought to be in the order of 1 in 1500 in Europeans, it is now thought to be in the order of 1 in 100 to 1 in 250. In the largest population screening study[1], 17 000 Italian school children, aged 6-15 years, were screened using a stepwise protocol with anti-gliadin antibodies (AGA), anti-endomysial antibodies (EMA), and finally duodenal biopsies in those who screened positive for these two serological tests. A prevalence rate of 1 in 184 was found. A Swedish study involving healthy blood donors found a prevalence of 1 in 256, confirmed by small bowel biopsy[2], and an American study[3] using EMA in blood donors found a rate of 1 in 250, although this was not confirmed by biopsy. In Ireland, the rates are even higher, with a reported prevalence rate of 1 in 122 determined in a screening study [4].

The incidence and prevalence of CD are therefore similar in populations with a similar genetic background. CD is thought to occur rarely in people from an Afro-Caribbean background, though there are reports of the condition in Asians from the Indian sub-continent[5]. CD is a familial condition, and the main risk factor for development of the condition is the presence of HLA DQ2 or DQ8. Most Northern European patients express the DQ2 heterodimer HLA-DQA1*0501 and DQB1*0201. Those who do not express this heterodimer, most commonly, have the HLA-DR4, DQ8 haplotype. In Italian and Tunisian patients there is also a significant association with DR53 heterodimers[6,7]. Further susceptibility genes, such as the CTLA-4 gene on chromosome 2q33[8], are thought to reside both inside and outside the HLA region and are currently being evaluated, although the disease is not expressed in the absence of the HLA genes. CD is thought to occur in 10%-15% of first degree relatives of probands[9], with 40% of HLA identical siblings being affected, and a concordance of 75% in monozygotic twins[10]. In some countries, such as Italy, first-degree relatives of patients with CD are screened routinely.

There are various serological screening tests available, which have different sensitivity and specificities. Circulating antibodies to gliadin were previously used for screening, but have largely been superseded due to their low specificity. Anti-endomysial antibodies (EMA) of the IgA class are considered highly specific markers of coeliac disease. Using human umbilical cord (HUC), the reported sensitivity is 90%, with a specificity of 99% in adults with

untreated coeliac disease (Table 1)[11]. However, this test is labour intensive and somewhat subjective, relying on the interpretation of a staining pattern on connective tissue, using a fluorescent microscope. The discovery of tissue transglutaminase (tTG) as the antigen for EMA[12], allowed the development of a simple ELISA to detect this antibody, the sensitivity and specificity of IgA tTG test are reported to be 94.5% and 93.7% respectively[13]. However, there are pitfalls in serological screening for CD. Selective IgA deficiency occurs in 2.6% of patients with CD[14], which is a 10-15 fold increase in prevalence of IgA deficiency over that in the general population. Testing for IgA antibodies only would cause false negative results and missed diagnoses. Additionally, a new sub-group of CD patients has recently been described, who develop only IgG class antibodies (specifically IgG1) to endomysium, in the absence of IgA class antibodies, and with normal quantities of total serum IgA[15].

We wished to establish an accurate screening protocol to assess the prevalence of undiagnosed coeliac disease in relatives of probands.

Table 1 Sensitivity, specificity and reproducibility of screening coeliac serology

	IgA-AGA	IgG-AGA	IgA-EMA
Sensitivity	83	86	90
Specificity	82	76	99

MATERIALS AND METHODS

Study subjects

We collected details of families where either one or more individuals were affected by CD. The families were identified either in the Gastroenterology Outpatient Clinic at St Thomas' Hospital, or by consultant colleagues at other hospitals. A further recruitment drive involved a short article and request for volunteers printed in the 'Crossed Grain' magazine, published by Coeliac UK for its members. In this way we were able to recruit a total of 151 families into the study. Of these 73 families had only one affected member and were referred to as single affected families, 78 families had more than one affected family member (range 2-7) and were referred to as multiply affected families. Full ethical approval was obtained from the Local Research Ethics Committee of St Thomas' Hospital (Ref. No.EC00/233). We recorded family relationships and collected blood for serology from as many relatives as were willing to consent. Serum was stored at -20℃. DNA was extracted from heparinized blood using the Nucleon BACC3 kit and stored at -20℃.

Controls

In order to set up the parameters for serological testing we first took a group of normal controls. These comprised laboratory staff and their relatives who were healthy and symptom-free. The age range was 24-60 years. Volunteers were questioned about the health of other family members. Those with any family history of gastrointestinal problems, diabetes and auto-immune thyroid disease were excluded from the study.

ELISA for anti-tissue transglutaminase antibodies

Two individuals with biopsy-confirmed CD and high titre for IgA and IgG-tTG antibody respectively were selected as positive controls. Sera of these individuals were made at 1:100 dilution, aliquoted and sterilised by gamma irradiation. ELISA was performed according to the method of Sulkanen[13]. Microtitre plates were coated with guinea pig liver tTG (Sigma T 5398), 1 μg per well in 100 μL of 0.05 mol/L tris buffered saline, with 5 mmol/L calcium chloride. The plates were left over night at 4℃, then washed three times with 0.05 mol/L TBS, 0.01 mol/L EDTA and 0.1% Tween 20 (TTBS). Test sera were diluted to 1:100 in TTBS, 100 μL of the test and positive control sera was added in duplicate to two plates. The plates were covered and incubated for 1 h at room temperature, then washed three times. Peroxidase-conjugated rabbit anti-human IgA (Dako P-0216) or anti-human IgG (Dako P-0214) was diluted to 1:2000 in TTBS, and added to the plates at 100 μL per well. This was incubated for 1 h at room temperature, and washed three times. The reaction was developed by adding OPD as substrate (Dako S2045), prepared according to the manufacturer's instructions. One hundred microlitres was placed in each well, and the plates were incubated in the dark at room temperature for 30 min. The plates were read on a Titertek Multiskan MCC/340 ELISA plate reader at 450 nm. The end point was reached when the IgA and IgG positive controls reached optical density of 1.2-1.3. The cut off value for a positive result was established as 0.3 for IgA and 0.325 for IgG. These values were calculated from the mean plus 2 standard deviations for our normal population. Individuals whose IgA-tTG was above the cut-off value were further investigated by IgA-EMA. Those without IgA-tTG antibodies, but with IgG-tTG antibodies went on to have total IgA quantification and IgG1-EMA. Those with negative results for both antibodies were considered negative for screening, and no further action was taken.

Indirect immunofluorescence for anti-endomysial antibody

The method used was described by Ladinser *et al*[16]. Human umbilical cord (HUC) was cut into 5-μm cryostat sections on 4 well coated slides. Each section was blocked with 100 μL of 1% BSA in PBS for 30 min. Test sera were diluted to a concentration of 1:5, and added to each well. Each experiment also contained a positive and a negative control. The sections were incubated for 30 min, and washed twice in a PBS bath. FITC-conjugated rabbit anti-human IgA (specific for alpha chains) immunofluorescent antibody (DAKO F0204) or FITC-conjugated mouse anti-human IgG1 (Sigma Monoclonal anti-human IgG1 clone 8c/6-39, product number F0767) was diluted to 1:40 using PBS. Fifty microlitres was added to each well and the sections were incubated at room temperature for 30 min in a humid chamber, and immersed in a PBS bath as before. Fluorescent mounting medium (DAKO, S3023) was added and the sections were examined immediately under a fluorescent microscope. The test was considered positive

Table 2 Overall results of coeliac screening for singly and multiply affected families

Type of family	Coeliacs (*n*)	Relatives (*n*)	Positive screenings	1st degree relatives	2nd degree relatives	Not related
Singly affected family	73	223	11	11	0	0
Multiply affected family	232	691	28	22	2	4

if the antibody stained the endomysium of umbilical arteries in a defined reticular pattern at a dilution of 1:5.

Total IgA quantification

This was performed by a competitive ELISA assay. Microtitre plates were coated with 100 μL of 2 μL/mL whole molecule human IgA (Harlan Sera-Lab PP-17-01) in PBS, and left overnight at 4℃. The plates were then washed three times in PBS with 0.05% Tween 20, blocked with 100 μL of 1% BSA, incubated at 37℃ for 1 h, and then drained.

The serum samples were diluted in peroxidase-conjugated rabbit anti-human IgA at a concentration of 1:4000. A standard curve was produced by diluting known amounts of human IgA in peroxidase-conjugated rabbit anti-human IgA. Dilutions and sera were pre-incubated for 30 min at room temperature, then added to the plates and incubated for a further 30 min. The plates were washed three times in PBS/Tween, the reaction was developed by adding OPD as a substrate (Dako S2045), prepared according to the manufacturer's instructions. One hundred microlitres was placed in each well. The plates were incubated in the dark at room temperature for 30 min and read on a Titertek Multiskan MCC/340 ELISA plate reader at 450 nm. The concentrations of IgA in the serum were calculated from the standard curve.

HLA-DQ typing

DNA was extracted from whole blood by the following protocol, using the Nucleon BACC kit (SL-8512). In brief, primer sequences were chosen to detect the presence of HLA-DR3, -DR7, -DR5 and -DR4, which are the most common haplotypes in patients with coeliac disease, being present in > 98% of European individuals with the condition. The products were loaded onto 1% agarose gel containing ethidium bromide. The gels were run for 22 min at 300 V, and visualised under UV light.

RESULTS

Nine hundred and thirteen serum samples were tested for anti-tTG antibodies, which were from first, second and third degree relatives, as well as some individuals who were not blood relatives of coeliac disease probands, including individuals with coeliac disease such as husbands or wives and their relatives.

IgA-tTG antibodies were found to be present in 60 individuals. When these were followed up with IgA-EMA, 36 were found to have IgA-EMA antibodies, and 24 individuals were deemed to be false positives. The individuals who were positive for IgA-EMA had IgA-tTG levels of 0.31 to 2.34, whilst those who were negative for IgA-EMA had IgA-tTG levels of 0.31-1.1. IgG-tTG antibodies in the absence of IgA-tTG antibodies were found in 194 individuals. Samples from all of these individuals were tested for IgG1-EMA. Of these, only 3 were found to be positive for IgG1 EMA. In total, 194 individuals had IgA quantification. Of these, only 2 out of the 3 IgG1 positive individuals were IgA deficient (Table 2).

Table 3 Single affected families-relationships of individuals to probands

Relationship	Number tested (*n*)	Number affected (*n*)	Percentage affected (%)
Mother	73	5	6.85
Father	73	4	5.48
Sibling	37	2	5.41
Child	18	0	0
Uncle/Aunt	5	0	0
Grandparent	6	0	0
Grandchild	2	0	0
Nephew/Niece	3	0	0
Husband/Wife	6	0	0

Of all the relatives in the single affected families, those newly diagnosed with coeliac disease are shown in Table 3. Five point four seven percent of first-degree relatives were found to have positive anti-endomysial antibodies, no second-degree relatives were found to have positive anti-endomysial antibodies. These data were analysed to give a percentage factor of those affected in each category of relative. There were not many children of individuals with CD in this group, as the proband was a child in the majority of volunteer families.

Of all the relatives in the multiply affected families, those newly diagnosed with coeliac disease are shown in Table 4. Five point forty-one percent of first degree relatives were found to have anti-endomysial antibodies, and 1.62% of second degree relatives we found to have positive coeliac antibodies. These data were analysed to give a percentage factor of those affected in each category of relative.

Four (2.52%) of the 159 individuals who were related only by marriage to the person with coeliac disease were found to have positive coeliac antibodies. This rate was significantly higher than would be expected for the general population (1%).

HLA-typing

The 39 individuals with positive anti-endomysial antibodies were further investigated by HLA-typing. Twenty-nine of these were successfully typed. Reasons for failure in the other 10 individuals included: inability to locate EDTA blood for extraction, poor DNA extraction and poor PCR or uninterpretable gels. Our HLA-typing revealed the same distribution of HLA-types, as would be expected in a population of patients with coeliac disease. The HLA types are shown in Table 5.

Table 4 Mutliply affected Families-Relationships of Individuals to probands

Relationship	Number tested (*n*)	Number affected (*n*)	Percentage affected (%)
Mother	51	3	5.88
Father	31	3	9.68
Sibling	165	9	5.4
Child	137	7	5.11
Uncle/Aunt	6	1	16.67
Grandparent	11	0	0
Grandchild	39	0	0
Nephew/Niece	67	1	1.49
3rd degree or more	24	0	0
Husband/Wife	159	4	2.52

Follow-up

We attempted to contact all those individuals with a positive screening result, to arrange a small intestinal biopsy. Thirty-five underwent a small intestinal biopsy. Of these, 32 were positive, having increased intra-epithelial lymphocytes with partial or sub-total villous atrophy. Three biopsies were reported as normal.

DISCUSSION

We used a two-tier screening system for coeliac disease. The initial anti-tTG ELISA test was used as a highly sensitive, cheap and simple initial screening test, rather than as a specific diagnostic test. The limits for a positive result were deliberately set low in order to avoid missing any cases, but this did have a major impact on the specificity, hence the number of positives were subsequently found to be EMA negative. While we would not propose this two-tier method for use in a diagnostic laboratory, we found it useful for rapid large scale screening, avoiding EMA testing on a great number of samples.

Our prevalence rates in relatives of probands with coeliac disease were significantly lower than those previously estimated by other groups (10%-15% for first degree relatives). This is perhaps surprising, since our families were recruited through voluntary self-referral. Thus, one may have expected increased rates, as suspicious symptoms may have encouraged some families to be volunteers. However, one would expect these lower rates in the multiply affected families since by definition, relatives of probands have already been diagnosed, and our figures only indicate the existence of undiagnosed cases. We used guinea-pig liver tissue transglutaminase as our detection antigen, on a cost basis. However, human recombinant tTg is now available, and might have given a higher rate of positive detection.

In the two IgA deficient individuals, one had a normal duodenal biopsy, while the other had an increase in intra-epithelial lymphocytes (IEL) only. The patient with increased IELs was investigated for ataxia when she joined our screening study. After having a gluten free diet (GFD) for 24 mo, her symptoms improved slightly.

It could be argued that volunteers in the study may be more likely to have been symptomatic, although we have no evidence for this. Our study raises the question whether we should screen first-degree relatives of patients with CD, since they carry a high-risk of being similarly affected. The screening method we proposed is sensitive, specific and non-invasive. The general well being of individuals with sub-clinical coeliac disease appears to improve on a GFD. It has been shown that there is a long-term health benefit to these people if a GFD is instituted with a reduction in the otherwise significantly increased mortality.

In conclusion, we propose the algorithm shown in Figure 1 for screening family members for coeliac disease, as we believe it is important that these individuals should be picked up, diagnosed and offered appropriate treatment.

Table 5 Results of HLA-typing for individuals with positive screening tests

HLA-type	DR3/DRx	DR3/DR7	DR5/DR7	DR4	DR3/DR4	DR4/DR7
Number	25	4	0	0	0	0
Percentage	86.2%	13.8%	0	0	0	0

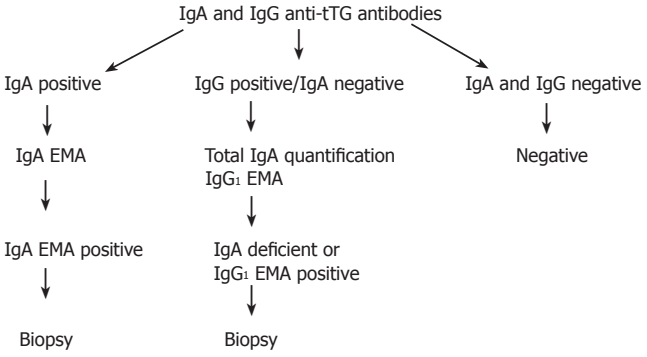

Figure 1 Algorithm for familial screening of relatives of probands affected with coeliac disease.

ACKNOWLEDGMENTS

The authors thank the following for financial support: Coeliac UK and Action Research (JSF), The German Federal Ministry of Education and Research (HJE) and the European Union (SJM).

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S- Editor Wang GP **L- Editor** Wang XL **E- Editor** Bai SH

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World J Gastroenterol 2006 December 28; 12(48): 7810-7814
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Management of digestive bleeding related to portal hypertension in cirrhotic patients: A French multicenter cross-sectional practice survey

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Received: 2006-10-10 Accepted: 2006-11-28

Abstract

AIM: To investigate the conformity of management practices of gastrointestinal hemorrhage in cirrhotic patients with relevant guidelines.

METHODS: A questionnaire on the management of digestive bleeding was completed for all consecutive cirrhotic patients admitted to 31 French hospitals.

RESULTS: One hundred and twenty-six bleeding events were recorded. It was the first bleeding episode in 79 patients (63%), of whom 40 (51%) had a prior diagnosis of cirrhosis and 25 (32%) had previously undergone an endoscopy. The bleeding episode was a recurrence in 46 patients (37%). The median time between onset and admission was 4 h, but exceeded 12 h in 42% of cases. There was an agreement between centers for early vasoactive drug administration (87% of cases), association with ligation (42%) more often than sclerosis (21%) at initial endoscopy, and antibiotic prophylaxis (64%). By contrast, prescription of beta-blockade alone or in combination (0 to 100%, $P = 0.003$) for secondary prophylaxis and lactulose (26% to 86%, $P = 0.04$), differed among centers.

CONCLUSION: In French hospitals, management of bleeding related to portal hypertension in cirrhotic patients is generally in keeping with the consensus. Broad variability still remains concerning beta-blockade use for secondary prophylaxis. Screening for esophageal varices, the use of antibiotic prophylaxis and patients information need to be improved.

Key words: Digestive bleeding; Portal hypertension; Cirrhosis; Evaluation studies

Ingrand P, Gournay J, Bernard P, Oberti F, Bernard-Chabert B, Pauwels A, Renard P, Bartoli E, Cadranel JF, Barbare JC, Ingrand I, Beauchant M, The Club Francophone pour l' Etude de l'Hypertension Portale. Management of digestive bleeding related to portal hypertension in cirrhotic patients: A French multicenter cross-sectional practice survey. *World J Gastroenterol* 2006; 12(48): 7810-7814

http://www.wjgnet.com/1007-9327/12/7810.asp

INTRODUCTION

Gastrointestinal hemorrhage is a major complication of cirrhosis, and several consensus conferences[1,2] have attempted to standardize its management. All the recommendations are based on results of meta analysis of randomized trials, which are designed to assess the efficacy of treatment modalities on survival. In fact, several recent reports suggest that better management has improved the prognosis of patients with variceal bleeding over the past four decades[3-6].

By contrast, the conformity of management practices with relevant guidelines in the field of portal hypertension has rarely been addressed. The few available studies[7-10]

have shown broad variability among centers and under-use of treatments designed to prevent bleeding. None of these surveys took into account the interval from bleeding onset to initial management, despite its prognostic significance[11].

The aim of this cross-sectional survey was to analyze French management practices for digestive bleeding linked to portal hypertension in patients with cirrhosis, and to evaluate their conformity with European guidelines[2].

MATERIALS AND METHODS

Patients and methods

This prospective questionnaire-based survey was conducted from 1 September to 30 November 2003 by gastroenterologists in 31 general and teaching hospitals located in five French regions (Aquitaine, Champagne-Ardennes, Pays de Loire, Picardie and Poitou-Charentes) and two counties (Oise and Val d'Oise). These seven sectors were non-randomly selected according to the following criteria: in each sector an investigator, member of the "Club Francophone d'Hypertension Portale", accepted to participate in the study and recruited all the hospitals covering the geographic area, including university and non-university hospitals. Patients were eligible if they had clinically or histologically documented cirrhosis of any cause, and if they had overt digestive bleeding related to portal hypertension presenting with hematemesis or melena. Patients were not eligible if bleeding was unrelated to portal hypertension, or if portal hypertension was not related to cirrhosis. The anonymous questionnaire included the patient's date of birth and home town, the mode of hospital admission, the interval between bleeding onset and initial management, the cause of bleeding, treatment, outcome 42 d after admission, the cause of cirrhosis, preventive measures started before and after the bleeding episode, transfer to a referral center, re-bleeding and its treatment, and complications of cirrhosis during the study period. Cirrhosis was graded on admission using the Child-Pugh score[12]. A single questionnaire was analyzed per patient, corresponding to the first bleeding event during the study period. Subsequent bleeding events were recorded but their management was not taken into account in this analysis. The study protocol was approved by the French Ethics Committee for the Treatment of Computerized Information concerning Research in Health Domain.

Statistics analysis

Data were recorded with the Microsoft Access database. All responses to the questionnaire were controlled and validated. Statistical analyses used SAS software version 8.2. Management practices were compared with the recommendations of the last consensus conference held before the survey, namely Baveno Ⅲ[2] and 95% confidence limits were calculated using the normal approximation or exact computation if not applicable. Differences in practices among the six geographic sectors (five regions and two combined counties) were analyzed with the generalized Fisher's exact test for qualitative variables, with a significance level of $P < 0.05$. Nonparametric Mann-Whitney test was used for quantitative variables. Bleeding control was defined as hemodynamic stability, without transfusion, 48 h after admission[2]. Hemorrhagic relapse was defined as any bleeding event occurring between 48 h and 42 d after admission. Mortality was evaluated 42 d after admission. In European studies published from 1993 to 1996, the estimated incidence of upper digestive bleeding was between $45/10^5$ and $143/10^5$ inhabitants, depending on the country, and esogastric varice rupture accounted for 5.0% to 13.7% of cases (incidence rate $4.0/10^5$ to $19.6/10^5$ inhabitants)[13-15]. Thus, it was predicted that a maximum of three months would be necessary to recruit at least 100 cases in the geographic area covered by the survey.

Table 1 Main characteristics of the patients

Characteristics	*n* = 126
Age (yr, median and range)	56 (32 to 83)
Sex (M/F)	103/23
Causes of cirrhosis [*n* (%)]	
- Alcoholism	101 (80%)
- Hepatitis virus (B, C)	11 (9%)
- Alcoholism plus another cause	4 (3%)
- Other causes	5 (4%)
- Unknown	5 (4%)
Child-Pugh class A/B/C (*n*)[1]	27/42/53

[1]Missing data: two incomplete files and two early deaths.

RESULTS

During the three-month study period, 135 patients were consecutively admitted in 31 hospitals. Nine patients were excluded from the analysis because the bleeding event was not linked to portal hypertension in 7 cases (gastric or duodenal ulcer in 5 cases, peptic esophagitis and hemorrhoidal bleeding in one case each). In the other two cases the portal hypertension was linked to hepatic metastases of breast and pancreatic primary tumors. The following analysis thus involved 126 patients.

The patients' main characteristics are shown in Table 1. Prophylaxes before admission are detailed in Table 2. Bleeding occurred at home in 100 cases (79%), and in hospitals in the other 26 cases. Forty-six patients (37%) arrived by mobile intensive care unit (MICU), either from home ($n = 37$) or from another hospital unit ($n = 9$). This mode of admission was evenly distributed among the participating centers ($P = 0.18$). Among the 100 patients who arrived from home, the median interval between onset and hospital admission was 4 h (1 to 80 h). This interval was significantly shorter among patients transported by MICU (median 3.4 h *vs* 5.2 h; $P = 0.049$). The interval was < 2 h in 18 cases, 2-6 h in 27, 6-12 h in 11, 12-24 h in 19 and > 24 h in 25. It did not differ between patients with first and subsequent bleeding events ($P = 0.14$), or between patients with and without documented cirrhosis ($P = 0.16$).

Table 3 shows the conformity to the Baveno Ⅲ consensus of management practices, and their variability among the participating centers. Ninety-five of the 100

Table 2 Previous bleeding and prophylaxis given before the index bleeding

Prophylaxis	*n* (%)
First bleeding event[1]	79 (63)
- Cirrhosis known before admission	40/79
- Prior endoscopy	25/40
- Prophylaxis before admission:	16/25
Esophageal varices stage 0-1:	0/7
Esophageal varices stage 2-3:	16/18
• Beta-blockade	12
• Ligation	2
• Ligation and beta-blockade	2
Recurrent bleeding	46 (37)
- Prophylaxis before admission:	
• None[2]	11 (24)
• Sclerosis	2 (4)
• Ligation	4 (9)
• Beta-blockade	13 (28)
• Ligation/sclerosis plus beta-blockade/nitrate derivative	16 (35)

[1]Missing data: one death shortly after admission; [2]Cirrhosis had not been diagnosed at the time of the previous hemorrhage in three patients.

patients who were admitted from home, underwent an endoscopy. The median interval between admission and endoscopy was 5 h (< 1-139 h), and was less than 12 h in 60% of cases. This interval was not influenced by blood transfusion ($P = 0.09$), the bleeding history ($P = 0.78$), or previously documented cirrhosis ($P = 0.29$). The median interval was 10 h when admission occurred between 10 PM and 7 AM, 4 h between 7 AM and 5 PM, and 13 h between 5 PM and 10 PM. Bleeding was due to esophageal varices in 89 cases (72%), gastric or ectopic varices in 13 cases (11%), and gastropathy linked to portal hypertension in 6 cases (5%); in 15 cases (12%) the endoscopy revealed mixed lesions combining occasionally erosive gastritis and signs of a possible variceal origin. Five of the 6 patients in whom bleeding was due to gastropathy alone received a vasoactive drug. Vasoactive drug therapy consisted mainly of octreotide (93% of cases); terlipressine was used alone in 6 cases and somatostatin in 2 cases. The use of vasoactive drugs did not differ among the geographic sectors ($P = 0.08$). The drugs were administered within a median of 2 h after admission (< 6 h in 66% of cases), for a mean of three days (1 to 6 d). None of the patients received emergency transjugular intrahepatic portosystemic shunting (TIPS), and a balloon tamponade was inserted in 8 (6%) patients. Tracheal intubation was performed in only 21 cases (17%), usually to facilitate an endoscopy ($n = 11$); this practice differed significantly among the geographic sectors (0 to 54%, $P < 0.0001$). Nasogastric tube was used in 57 patients (46%), usually for gastric lavage before an endoscopy (significant difference among geographic sectors; 25% to 86%, $P = 0.0002$). Data concerning antibiotics and lactulose prescription are given in Table 3.

Bleeding was controlled within 48 h after admission in 99 patients (80%). Twenty-one patients re-bled, a median of 16 d (d 3 to 40) after admission; they usually received a combination of endoscopic treatment and vasoactive drugs. TIPS was used in four patients, and one patient had a surgical portocaval anastomosis. Five patients were transferred to a referral center. In 94 patients evaluable, after exclusion of deaths, the main prophylactic treatments were a combination of beta-blockade and ligation in 31 patients, beta-blockade alone in 29 patients, ligation alone in 18 patients, endoscopic sclerosis in 4 patients and 3 patients received no prophylaxis. Prescription of beta-blockade, alone or in combination, was significantly different among geographic sectors ($P = 0.003$).

Outcome on d 42 was unavailable in 9 cases. Ninety-one (78%) were alive on d 42. Six of the 26 deaths occurred in the first 48 h. The main causes of death were hemorrhage, liver failure, multiorgan failure, shock, and hepatorenal syndrome. Fifteen patients had hepatocellular carcinoma, and three of them died before d 42. The main in-hospital complications were hepatic encephalopathy ($n = 14$), hepatorenal syndrome ($n = 6$), and bacterial infections ($n = 7$).

DISCUSSION

This survey shows that the management of digestive bleeding in cirrhotic patients in France generally complies with the Baveno Ⅲ international consensus statement issued three years ago. However, certain practices differed among the participating centers, and some recommendations should be applied more systematically. This is not surprising since many practitioners are involved in the management of cirrhotic patients and most of them are not hepatology experts. Our study was not designed to compare the outcomes of patients according to the physician's compliance with the recommendations. In our study, the outcome was as favorable as in recent publications[4,5]. However, improvement in mortality has rarely been observed in randomized trials, and this benefit was demonstrated only in meta analysis, suggesting that many confounding factors are involved.

First bleeding events revealed the presence of cirrhosis in half the patients. One-third of the patients with a prior diagnosis of cirrhosis had not had endoscopic screening for large esophageal varices and did not therefore receive preventive therapy. Esophageal varices are of a recognized prognostic value in this setting[16,17], and both beta-blockade[18] and endoscopic ligation[19] are known to reduce the bleeding risk. Our results are similar to those of US practice surveys. Arguedas *et al*[7] reported that only one-half of cirrhotic patients referred for liver transplantation had endoscopic screening for varices. Sorbi *et al*[8], in a survey undertaken in 1997 in the United States, also noted that primary prophylaxis was under-used, as only 20% to 30% of patients received beta-blockade before the index bleeding event. Following the publication of the 1997 guidelines of the American Board of Gastroenterology, Zaman *et al*[10] found that 54% of gastroenterologists claimed they followed recommendations to screen for and treat large varices in patients with no history of bleeding, representing a three-fold increase compared to the same survey prior to the publication of the recommendations.

Admission to clinical centers remains too late in many cases (> 12 h in nearly 40% of patients), even though the general French population is no more than one hour from a hospital. The interval between onset and initial

Table 3 Management of the bleeding event in 126 consecutive patients: conformity with the Baveno Ⅲ consensus and regional variability

Baveno Ⅲ guidelines	*n* (%) of cases complying with the guidelines (95% confidence limits)	Variability among sectors (range, *P*[a])
Endoscopy within 12 h after admission from home	60 (60%) (50%-70%)	52% to 71% *P* = 0.85
Blood restitution to maintain hematocrit at 25% to 30%	84 (89%) (81%-95%) (*n* = 94 patients transfused)	ND
Early vasoactive drug infusion	110 (87%) (80%-92%) Median time after admission: 2 h	71% to 100% *P* = 0.081
Endoscopic treatment: ligation or sclerosis	- Ligation 52 (42%) (33%-51%) - Sclerosis 26 (21%) (14%-29%)	29% to 61% *P* = 0.17 7% to 43% *P* = 0.29
Antibiotic prophylaxis	81 (64%) (55%-73%) Prophylaxis 87%/infection 13%	50% to 75% *P* = 0.41
Lactulose administration	50 (40%) (32%-49%) Prophylaxis 57%/encephalopathy 43%	26% to 86% *P* = 0.041
Prophylaxis of rebleeding: beta blockers or ligation	- Beta-blockers alone 31% (22%-41%) - Ligation and beta-blockers 33% (24%-43%) - Ligation alone 19% (12%-29%) - Others or none 17% (10%-26%)	0 to 45% 16% to 100% 0 to 37% 0 to 44% *P* = 0.0003

ND: variability not assessed. [a]Generalized Fisher's exact test of the homogeneity in practices among the six geographic sectors (five regions and two combined counties).

management is not shorter in patients with a history of bleeding or with known cirrhosis, suggesting that they are poorly informed of the risk of variceal bleeding and the need for early hospital admission. Although overall survival in this survey was better than previous, and similar to that in recent publications[5,6], fatal outcome is still closely related to failed bleeding control or to early rebleeding[11]. Early resuscitation is firmly recommended[20]. We recently showed that about one-quarter of deaths occur very early after bleeding onset, mainly before hospital admission[21]. Levacher *et al*[22] also reported that early terlipressin administration (en route to hospital) significantly improved the prognosis. This should be taken into account at the forthcoming consensus conferences.

Contrary to recommendations, an endoscopy was performed more than 12 h after admission in one-third of patients admitted from home in this survey. However, early use of vasoactive drug therapy in nearly all the patients, as recommended, suggests that initial bleeding control allowed an endoscopy to be deferred, particularly among patients admitted in the evenings. Conversely, one-third of patients did not receive antibiotics, which should have been routinely considered[2]. Indeed, antibiotics can prevent infections and rebleeding, and thereby improve survival[23]. In our survey, lactulose was only prescribed to about 40% of patients, and there were significant differences among the participating centers with respect to this practice. This is not surprising since the efficacy of lactulose in preventing encephalopathy has not been clearly demonstrated.

Regarding prophylactic measures, beta-blockade was extensively used for primary prevention in patients with large varices, in keeping with the consensus, however, secondary prevention in one third of patients consisted of a combination of beta-blockade and endoscopic ligation, even though this treatment had not been shown at the time of Baveno Ⅲ to be more effective than ligation or beta-blockade alone[2]. Practices differed significantly between centers regarding secondary prophylaxis, and the combined treatment was finally accepted in 2005 consensus[1]. This study was not designed to investigate the cause and origins of lack of adherence to guidelines, which is a worldwide problem. Many factors may be involved, including patient information and behavior, local organization of health care, formation of practitioners especially in non-specialized emergency units. This question needs to be addressed in the future.

In conclusion, while French practices are generally in line with the consensus statement, there is significant room for improvement in the diagnosis of cirrhosis and in primary bleeding prevention. However, these results show that cirrhotic patients are poorly informed of the clinical signs and gravity of bleeding, and of the need for rapid treatment by a specialized team. Antibiotics are under-used, and this calls for better information of physicians who manage such patients in intensive care units.

ACKNOWLEDGMENTS

The authors thank David Young for translating the French manuscript.

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COMMENTS

Background

Gastrointestinal hemorrhage is a major complication of cirrhosis. Prognosis improved over the past four decades in relation with a better management of patients with variceal bleeding. Variability and conformity of practices with relevant guidelines have rarely been addressed. Previous US practice surveys reported underuse of esophageal varices screening and primary prophylaxis with beta-blockers.

Research frontiers

This article deals with evaluative epidemiology of medical practices. The lack of adherence to guidelines is a worldwide problem.

Innovations and breakthroughs

Major concerns for improvement: improving the endoscopic screening of esophageal varices, patient information about the clinical signs and gravity of bleeding, shortening the delay from bleeding to admission and endoscopy, and generalizing antibiotic prophylaxis. Lactulose administration and prophylaxis of rebleeding were highly variable practices among sectors.

Applications

These results support the need for active promotion of international guidelines focused on information of physicians who manage such patients and encouraging them to criticize their own practice.

Peer review

This is an interesting cross-sectional, descriptive study of treatment related to adherence to international guidelines in a French mixture of academic and non-academic hospitals. However, this work did not analyse possible causes to explain such behavior.

S- Editor Liu Y **L- Editor** Zhu LH **E- Editor** Liu WF

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World J Gastroenterol 2006 December 28; 12(48): 7815-7820
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

H pylori infection and systemic antibodies to CagA and heat shock protein 60 in patients with coronary heart disease

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Supported by a grant from the University of Siena, PAR 2004 "*H pylori* infection, hosts' aplotypes of inflammatory cytokines and the risk of ischemic heart disease"
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Received: 2006-10-05 Accepted: 2006-11-30

Abstract

AIM: To determine the overall prevalence of *H pylori* and CagA positive *H pylori* infection and the prevalence of other bacterial and viral causes of chronic infection in patients with coronary heart disease (CHD), and the potential role of anti-heat-shock protein 60 (Hsp60) antibody response to these proteins in increasing the risk of CHD development.

METHODS: Eighty patients with CHD and 160 controls were employed. We also compared the levels of anti-heat-shock protein 60 (Hsp60) antibodies in the two groups. The *H pylori* infection and the CagA status were determined serologically, using commercially available enzyme-linked immunosorbent assays (ELISA), and a Western blotting method developed in our laboratory. Systemic antibodies to Hsp60 were determined by a sandwich ELISA, using a polyclonal antibody to Hsp60 to sensitise polystyrene plates and a commercially available human Hsp60 as an antigen.

RESULTS: The overall prevalence of *H pylori* infection was 78.7% (n = 63) in patients and 76.2% (n = 122) in controls (P = 0.07). Patients infected by CagA-positive (CagA$^+$) *H pylori* strains were 71.4% (n = 45) *vs* 52.4% of infected controls (P = 0.030, OR = 2.27). Systemic levels of IgG to Hsp60 were increased in *H pylori*-negative patients compared with uninfected controls (P < 0.001) and CagA-positive infected patients compared with CagA-positive infected controls (P = 0.007).

CONCLUSION: CagA positive *H pylori* infection may concur to the development of CHD; high levels of anti-Hsp60 antibodies may constitute a marker and/or a concomitant pathogenic factor of the disease.

Key words: *H pylori*; Coronary heart disease; CagA protein; Heat shock protein 60; Antibody response

Lenzi C, Palazzuoli A, Giordano N, Alegente G, Gonnelli C, Campagna MS, Santucci A, Sozzi M, Papakostas P, Rollo F, Nuti R, Figura N. *H pylori* infection and systemic antibodies to CagA and heat shock protein 60 in patients with coronary heart disease. *World J Gastroenterol* 2006; 12(48): 7815-7820

http://www.wjgnet.com/1007-9327/12/7815.asp

INTRODUCTION

Atherosclerosis-related diseases -particularly coronary heart disease (CHD)- are a leading cause of death and disability in most developed countries. Many epidemiological studies have shown a strong relationship between CHD and chronic bacterial and viral infections, suggesting a primary role of inflammatory diseases in the pathogenesis of vascular cardiac disorders[1,2]. Infectious agents may cause a spectrum of systemic effects and induce atherosclerosis in several different ways. For instance, by increasing the production of circulating cytokines (interleukin-1 [IL-1] and interleukin-6 [IL-6]), through the generation of acute-phase reactants (white blood cells and C reactive protein) and the stimulation of immune-mediated responses, such as the production of antibodies targeted to the invading pathogens, *etc*[3]. Several authors[4] have also reported that infections might stimulate smooth muscle cell proliferation and migration and lipid accumulation; apoptosis of endothelial cells can be inhibited and many procoagulant effects could be produced[4].

H pylori infection is one of the most widely spread in-

fectious diseases in human[5]. This microorganism infects half the world population and causes chronic gastritis. The disease usually lasts for the entire host's life and constitutes a main risk determinant of peptic ulcer and gastric neoplasia[6,7]. The infection elicits a chronic humoral and cellular inflammatory response, stimulates an increase of polymorphs and basophils[8] and elevates the local and systemic concentrations of vasoactive cytokines[9], whose effects may not be confined to the digestive tract[10].

Recent epidemiological surveys have indicated that *H pylori* infection may be associated with atherosclerotic vascular diseases[11], although it is still disputed whether this infection increases the risk of CHD[12-14]. Some studies have shown an increased risk of CHD in patients with a systemic immune response to heat shock proteins (Hsps)[15]. Hsps are families of highly conserved proteins that share wide homologies of sequence among different species, ranging from bacteria to human beings[16,17]. They are induced or up-regulated in cells exposed to sudden elevations in temperature, but are also synthesized in large numbers when cells are exposed to stressful stimuli such as inflammation, infections, mechanical stress, hypoxia and oxidizing agents[16,17]. They play a fundamental role in the growth of bacteria at all temperatures and their production could represent an essential mechanism of cell protection against different noxae[17,18]. *H pylori* produces two main Hsps, a groEs-like HspA with a mass of 13 kDa, and a groEL-like HspB with a mass of 54-60 kDa[19,20]. Both proteins stimulate a specific systemic antibody response and, due to the high sequence homology of Hsps, it is highly possible that they can trigger an autoimmune response directed against the bacterial proteins and also to human tissues expressing Hsps, including vascular endothelial cells[20,21]. The aim of the present study was to determine the prevalence of anti-Hsp antibodies in patients with CHD and controls and to identify the potential role of an antibody response to these proteins in increasing the risk of CHD development. We tested serum samples for the overall prevalence of *H pylori* and CagA positive *H pylori* infection, and for antibodies to the other bacterial and viral causes of chronic infection that are recognised determinants of CHD risk development. Our results suggest that CagA positive *H pylori* infection may concur to the development of CHD and that high levels of anti-Hsp antibodies may constitute a marker and/or a pathogenic factor of the disease.

MATERIALS AND METHODS

Patients and controls

We studied 80 consecutive patients with stable angina; their mean age was 65 years (range 45 to 75 years). Patients were admitted to this Institute for evaluation by clinical history, physical examinations, heart echography, and basal and exercise ECGs. Patients were enrolled if they showed signs or symptoms of angina at exercise ECG; an ST segment depression more than 2 mm was considered positive. As control, we enrolled 160 age- and gender-matched patients, who came from the same socio-economic background and were hospitalised in the same Institute for diseases other than CHD, vascular diseases, dyspeptic and liver disorders, hematological diseases, and thyroid abnormalities. Their mean age was 64.5 years (range 43 to 75 years). Patients and controls had not taken antibiotics potentially active against *H pylori* in the last three months. Both patients and controls gave their written informed consent.

Determination of H pylori infection and CagA status

The *H pylori* infectious status was determined serologically using a commercially available enzyme-linked immunosorbent assay with a sensitivity and specificity of 96% *ca.* (*Helicobacter pylori* IgG, Diesse, Monteriggioni, Siena, Italy). *H pylori* infectious status was confirmed by Western blotting (WB). WB was also used to detect antibodies to *H pylori* CagA. Briefly, a whole cell suspension of *H pylori* CCUG 17874 (a CagA-positive and cytotoxic strain) was denatured in Laemmli's buffer at 100℃ for 5 min and electrophoresed in a 10% polyacrylamide gel with sodium dodecylsulphate. The resolved proteins were transferred electrophoretically onto nitrocellulose membranes, and the free sites were saturated with 3% skim milk in phosphate buffered saline (PBS) pH 7.4 containing 0.1% Triton X (PMT). Afterward, strips were cut and immunoblotted with serum samples diluted 1:100 in PMT for immunoglobulin G (IgG). After overnight incubation at room temperatures, strips were washed three times with PMT, and a peroxidase labelled antibody to human IgG, diluted in PMT 1:2000 (Sigma Che. Co., Milan), was added and incubated at room temperatures for 90 min. Strips were washed three times with PMT, once with PBS-Triton X, and twice with Tris buffer 0.05 mol/L pH 6.8. The reaction was visualised by addition of the substrate (H_2O_2 in a solution of 4-chloro-1-naphthol in Tris buffer 0.05 M pH 6.8). The reaction was stopped with water. The presence of more than six bands of reaction indicated an infection. As positive controls, anti-CagA and anti-Hsp rabbit polyclonal antibodies (kindly given by R. Rappuoli, Novartis, Siena) were used.

Determination of anti-Hsp60 antibodies

Antibodies to Hsp60 were determined by an ELISA, using a commercially available human Hsp60 (Sigma Che. Co., Milan, Italy). In preliminary tests, we determined the working concentrations of Hsp60 with the aid of a pool of human serum samples, which contained antibodies to *H pylori* HspB (54-60 kDa), as detected on WB. Briefly, we sensitised each well of polystyrene microtiter plates with 150 μL of an anti-polyclonal *H pylori* HspB antibody raised in rabbits, diluted 1:50 in PBS pH 7.4. After one hour of incubation at 37℃, we washed the plates three times with PBS containing 0.05% Tween 20 (PBST) and 2% bovine serum albumin (BSA). Then, we added to each well 2.5 μg of Hsp contained in 100 μL of PBS-BSA (this amount of Hsp was determined in preliminary tests). After one hour of incubation at 37℃ and three washes with PBS-T-BSA, we added 100 μL of each serum samples, both from patients and controls, diluted 1:50 in PBS-BSA. Plates were incubated at 37℃ for one hour, then they were washed

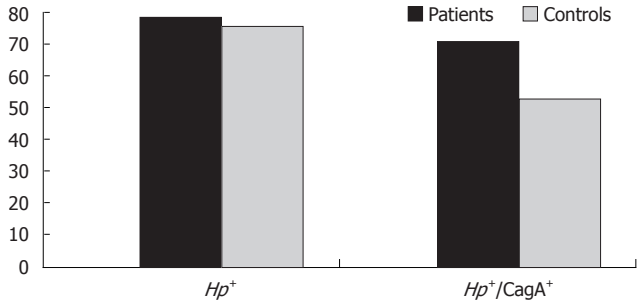

Figure 1 Prevalence of overall *H pylori* and CagA+ *H pylori* infection in patients and controls.

three times and 100 μL of an anti-human immunoglobulin G (IgG) (Sigma Che. Co., Milan, Italy), labelled with peroxidase, diluted 1:2000, was added to each well. After incubation, we added to each well 50 μL of the substrate (a solution of tetramethylbenzidine dihydrochloride in 0.1 mol/L phosphate-citrate buffer pH 5.0, containing 0.03% sodium perborate and 0.01% of 30% hydrogen peroxide). Incubation was carried out at room temperatures in the dark for 30 min. The reaction was stopped by the addition of 50 μL of 2 mol/L sulphuric acid and read spectrophotometrically at 450 nm. All tests were performed in triplicate and levels of antibodies were expressed in optical density (OD). As a control, we used an anti-Hsp60 polyclonal serum raised in rabbits tested at several dilutions. The intra-tests and inter-tests deviations were lower than or equal to 10%, and lower than or equal to 15%, respectively.

Determination of other pathogens

Antibodies against other putative causes of chronic infections were determined by the following commercially available kits: *Chlamydia pneumoniae* (CFT-MAT Chlamydia, Diesse, Monteriggioni, Italy); *Mycoplasma pneumoniae* (CFF-MAT Mycoplasma, Diesse, Monteriggioni, Italy); cytomegalovirus (ENZIGNOST CMV IgG, Behering, Milan, Italy); *herpes simplex* virus (ENZIGNOST HSV IgG, Behering, Milan, Italy); E-B virus (ENZIGNOST HSV IgG, Behering, Milan, Italy).

Statistical analysis

The differences in the prevalence of infection by the various pathogens were compared using the chi-square test with the Yates's correction. The mean levels of anti-urease antibodies were compared using the *t*-test for independent samples, utilising the software Primit.Exe, version 3.0.1. *P* values < 0.05 were considered significant.

RESULTS

Overall prevalence of H pylori infection

We determined the prevalence of *H pylori* infection between patients and controls to verify the hypothesis that such an infection could increase the risk of CHD. The mean age of patients was 65 years (range 45 to 75 years); controls had a similar mean age of 64.5 years (range 43 to 75 years). The overall prevalence of *H pylori* infection was 78.7% ($n = 63$) in patients and 76.2% ($n = 122$) in controls ($P = 0.07$).

Table 1 Prevalence of infection by pathogens other than *H pylori* in patients and controls *n* (%)

Group	*C. pneumoniae*	CMV	HSV-1	EBV	*M. pneumoniae*
Patients (80)	63 (78.7)	75 (93.5)	77 (96.2)	74 (92.5)	37 (46.2)
Controls (160)	110 (68.7)	145 (90.6)	140 (87.5)	148 (92.5)	62 (38.7)

Determination of CagA status in infected patients and controls

Recent studies have shown that infection by strains that express CagA protein induces increased levels of local and systemic cytokines that could contribute to the damage of the cardiovascular system. We therefore determined the seroprevalence of CagA seropositivity in patients and controls. Patients infected by CagA-positive (CagA+) *H pylori* strains were 71.4% ($n = 45$) *vs* 52.4% of infected controls ($P = 0.030$, OR = 2.27; 95% CI 1.0-5.1) (Figure 1).

Prevalence of infections by pathogens other than H pylori

Since it is well-known that many pathogens could contribute to the genesis of a chronic systemic inflammatory status, we determined the seroprevalence of the most common infectious agents that might increase the risk of CHD. We found that the majority of both patients and controls were seropositive for *C. pneumoniae,* cytomegalovirus*, herpes simplex* virus and Epstein-Barr virus, while 46.2% of patients and 38.7% of controls had anti-*M. pneumoniae* antibodies (Table 1). No statistically significant difference was found in the prevalence of infections by the different pathogens in patients and controls (data not shown).

Determination of anti-Hsp60 antibodies in patients and controls

Hsps are a family of well-conserved proteins and Hsp60, in particular, is widely shared by *H pylori* and eukaryotic cells. As antibodies to Hsps are found at high titers in cardiovascular disorders, we compared the levels of anti-Hsp60 antibodies in patients and controls. Levels of antibodies to Hsp60 were significantly increased in *H pylori*-negative (*Hp*-) patients, compared with those in *H pylori*-negative controls (341.5 ± 159.6 *vs* 197.6 ± 44.4; $P < 0.001$, 95% CI 66.4-221.3) (Figure 2); levels of antibodies to Hsp60 in CagA+ patients were higher than in CagA+ controls (418.8 ± 144.2 *vs* 317.2 ± 175.6; $P = 0.007$, 95% CI 28.8-174.3), but were not significantly higher than in *Hp*+/CagA- patients (350.2 ± 169.1; $P = 0.110$) and in *Hp*- patients (341.5 ± 159.6; $P = 0.072$) (Figure 2). Levels of antibodies to Hsp60 in CagA+ controls (317.2 ± 175.6)

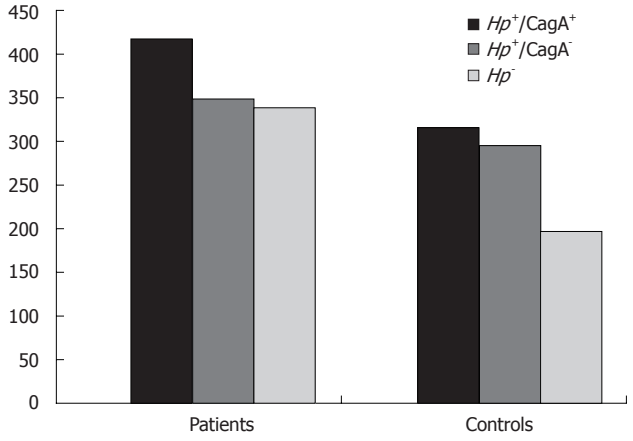

Figure 2 Mean levels (in optical density) of anti-Hsp60 systemic antibodies in patients and controls.

and Hp^+/CagA$^-$ controls (297.1 ± 80.82) were higher than in Hp^- controls (197.6 ± 44.42) ($P = 0.006$, and $P < 0.001$, respectively) (Figure 2).

DISCUSSION

In the present study we have observed an increased prevalence of CagA$^+$ *H pylori* infection, as well as increased levels of antibodies to Hsp60 in patients with CHD, compared with controls. Because of the notion that atherosclerosis may be regarded as an inflammatory process and that the presence of a chronic systemic inflammatory status is a strong risk factor for the development of coronary artery disease and ischemic stroke, we need to identify the cause of inflammation. In our study, almost all patients and controls were seropositive for pathogens other than *H pylori*, suggesting that these agents of chronic infection have a minor role (if any), at least in our area, in the development of cardiovascular disorders. *H pylori* is the logical and most important candidate for this role as a cause of the low-grade, persistent inflammatory stimulation induced by the infection, i.e. the vascular damage could be an indirect effect of systemic inflammatory mediators stimulated by the local mucosal inflammation, which may affect homeostasis. Recent epidemiological surveys have indicated that *H pylori* infection may be associated with atherosclerosis in different districts, but it is still disputed whether this infection increases the risk of CHD[11,12,14,21]. The increased levels of cytokines induced by infections can lead to changes in endothelial cell function by recruiting monocytes and T cell lymphocytes into the vessel walls; as a consequence, the local inflammatory responses may be exacerbated, even in the absence of resident pathogens. In addition to promoting atherosclerosis, infections can also trigger acute coronary events, such as plaque rupture[12]. Mendall *et al*[11] reported an association between *H pylori* infection and CHD, however, such an observation is still questioned[12-14,22]. A possible reason for contradictory results may include the variable circulation in different areas of *H pylori* strains endowed with an increased inflammatory potential, i.e. those possessing a genomic insertion called *cag*[23]. Patients infected by highly virulent *H pylori* strains can be easily identified, because CagA, the protein encoded by *cagA*, one of the *cag* genes, is always expressed[23,24]. CagA is strongly immunogenic and specific systemic antibodies can be determined by simple methods. *H pylori* is not a clonal pathogen. Although strains from unrelated cases of infection are all genomically diverse, most clinical isolates can be grouped into two types, according to whether they possess or not the pathogenicity island *cag*, a genomic region that encodes proteins involved in virulence such as *cagA*[23]. The importance of the infection by *H pylori* carrying the *cag* insertion has recently been confirmed by the observation that patients with CHD are more likely infected by CagA positive strains and have a more severe clinical picture of the disease[1,2,25]. The increased risk of CHD, to which patients infected by CagA-positive *H pylori* are exposed, may be attributed to the intensified inflammatory potential of such organisms[23]. Thus, the infectious status could determine elevated systemic levels of tumour necrosis factor-alfa, IL-1 beta, IL-6 and interleukin-8 and these cytokines may exert a deleterious activity against vascular endothelial cells. The chronic local inflammatory response to *H pylori* infection may also have repercussions for the whole organism[8,9]. Systemic indices of inflammation, such as levels of polymorphs and basophils, can be increased in individuals infected by *H pylori* and many vasoactive substances like cytokines, produced locally to fight the bacteria, may reach the blood stream and promote a chronic systemic inflammatory status of low degree, which may contribute to injury of other organs, different and far from the stomach[8-11].

Another explanation for contradictory results in experimental and clinical studies, pointing to find out a real association between *Hp* and CHD, may reside in the different inclusion criteria of patients and controls employed in different studies and the strong association of *H pylori* infection with confounding factors, such as age and social class. In order to avoid these variables we have enrolled both patients and controls of the same gender, age and coming from the same social background. The infection by CagA-positive *H pylori* cannot directly induce coronary atherosclerosis and need, most probably, the presence of other co-factors capable of inducing the onset and evolution of ischaemic heart disease. Hsps are highly conserved, immunogenic molecules whose cellular levels are raised by heat, inflammatory mediators and other forms of physiological stress[16,17]. Their most important function is to enhance cellular survival under physiologically stressful conditions, since they have been recognized as molecular chaperones and helpful in correct protein folding and oligomeric assembly[16-18]. Although Hsps are normally intracellular proteins, marked stress-induced overexpression may lead to their presentation on the cell surface, stimulating an autoimmune reaction, and thereby representing an important marker of inflammation. The observation that almost all patients with gastric carcinoma have systemic antibodies to *H pylori* HspB may support such observations[26]. According to the hypothesis of Wick *et al*[27], the association between high levels of anti-Hsp antibodies and

atherosclerotic vascular disease is due to an autoimmune reaction to endothelial cells that express high levels of Hsp in response to stressful stimuli, like oxyded LDL, free radicals, local infections, cytokines or hemodynamic stress. In the present study, we confirmed that the increased anti-Hsp60 immune response observed in patients cannot be attributed to chronic infections by pathogens other than *H pylori*, since their prevalence in patients was similar to that in controls. On the contrary, the infection by $CagA^+$ *H pylori* strains increased the levels of anti-Hsp60 antibodies both in patients and controls; however, although in patients the difference was not statistically significant, in controls such a difference was significant, suggesting that a relationship between chronic *H pylori* infection and development of antibodies to Hsp60 cannot be excluded. Another explanation could consist in the possibility that the inflammatory response triggered by the infection, together with putative toxic substances secreted by bacteria, alters the epithelial Hsp to such a degree that the patient' s immune system loses the immune tolerance to self chaperon and starts producing autoantibodies that cross-react with *H pylori* Hsps. Latif *et al*[28] have identified a strong homology between cardiac myosin heavy chain and Hsp60, suggesting that a cross-reactivity between similar epitope motifs may contribute to autoimmunity; circulating anti-Hsp antibodies may be involved in an autoimmune reaction to myocytes or endothelial cells, respectively, that have expressed Hsps due to stress.

Considering the importance of CHD in the industrialized world as a main cause of illness and since *H pylori* infection (even by CagA-positive strains) can be easily eradicated by specific treatments, the accurate definition of this new risk factor may lead to novel strategies for the prevention of ischemic heart disease.

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S- Editor Wang J **L- Editor** Zhu LH **E- Editor** Ma WH

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World J Gastroenterol 2006 December 28; 12(48): 7821-7825
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Non invasive evaluation of liver fibrosis in paediatric patients with nonalcoholic steatohepatitis

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Received: 2006-09-17 Accepted: 2006-11-30

Abstract

AIM: To identify the independent predictors of hepatic fibrosis in 69 children with nonalcoholic steatohepatitis (NASH) due to nonalcoholic fatty liver disease (NAFLD).

METHODS: All patients with clinically suspected NASH underwent liver biopsy as a confirmatory test. The following clinical and biochemical variables at baseline were examined as likely predictors of fibrosis at histology: age, body mass index (BMI), systolic blood pressure (SBP), dyastolic blood pressure (DBP), fasting glucose, fasting insulin, homeostatic model assessment for insulin resistence (HOMA-IR), cholesterol, tryglicerides, alanine aminotransferase (ALT), aspartate aminotransferase (AST), AST/ALT ratio, gamma glutamil transferase (GT), platelet count, prothrombin time (PT).

RESULTS: At histology 28 (40.6%) patients had no fibrosis and 41 (59.4%) had mild to bridging fibrosis. At multivariate analysis, BMI > 26.3 was the only independent predictor of fibrosis (OR = 5.85, 95% CI = 1.6-21).

CONCLUSION: BMI helps identify children with NASH who might have fibrotic deposition in the liver.

Key words: Nonalcoholic steatohepatitis; Obesity; Body mass index; Liver fibrosis; Non invasive diagnosis

Iacobellis A, Marcellini M, Andriulli A, Perri F, Leandro G, Devito R, Nobili V. Non invasive evaluation of liver fibrosis in paediatric patients with nonalcoholic steatohepatitis. *World J Gastroenterol* 2006; 12(48): 7821-7825

http://www.wjgnet.com/1007-9327/12/7821.asp

INTRODUCTION

With the current epidemic prevalence of obesity and diabetes mellitus in the general population[1], nonalcoholic fatty liver disease (NAFLD) has become the most common cause of chronic liver disease in many countries[2,3]. The characteristic histological features of NAFLD may range from a bland hepatic steatosis to hepatocellular damage plus inflammation with or without fibrosis known as nonalcoholic steatohepatitis (NASH)[4,5]. Paralleling the increasing prevalence of obesity and type 2 diabetes in the pediatric population, NAFLD especially its more severe histological form-NASH, is expected to become one of the most common causes of end-stage liver disease in both children and young adults. In this population, histological confirmation remains controversial due to the cost of liver biopsy and the complications directly related to the procedure or to the sedation, reported to be higher (up to 18%) in infants than in adults[6]. Furthermore, there is no proven therapy for NASH that could justify paired biopsies to compare histology with baseline features.

A first step for the clinician is to select children at risk of progressive liver disease from those with steatosis alone, and the secondary step is to identify in the former possibly by non invasive diagnosis of those with liver fibrosis. Raised alanine aminotransferase levels (ALT) have been shown to correlate best with a diagnosis of steatohepatitis (95% CI = 3.1-23.5, $P < 0.001$), but the other two factors, namely insuline resistence index (IR > 5.0, 95% CI = 3.4 -26, $P < 0.001$) and hypertension (> 140/90, 95% CI = 2.0-13.5, $P = 0.001$), are also found to have significant independent predictive effects[7]. The presence of at least two of the three factors provides the best combination of sensitivity (0.8) and specificity (0.89) for predicting NASH[7].

Non invasive diagnosis of liver fibrosis has been extensively evaluated in adult population with NAFLD[8-11]. Obesity, hypertension, male gender, hyperdyslipemia, and insulin resistance have been reported to be independent predictors of advanced fibrosis. In contrast, in paediatric population data are lacking and liver biopsy is still considered the only reliable tool for diagnosing histological

features.

The aim of this study was to identify the independent predictors of fibrosis in children with NAFLD by selecting groups of children at a higher risk of progressive liver damage (NASH population). Clinical and/or biochemical parameters routinely performed in clinical practice that can predict abnormal liver histology were tested.

MATERIALS AND METHODS

The study protocol was conformed to the ethical guidelines of the 1975 Declaration of Helsinki and performed according to the recommendations of the Ethics Committee of Children's Hospital and Research Institute Bambino Gesù in Rome, Italy. Informed consent was directly obtained from each parent or responsible guardian.

Patients

Sixty-nine untreated consecutive children (49 males and 20 females) seen at our institution from June 2001 to April 2003 were included in this study. All patients underwent evaluation for persistently elevated serum aminotransferase levels associated with diffusely echogenic liver on imaging studies suggestive of fatty infiltration. The diagnosis of NAFLD was confirmed by a percutaneous liver biopsy in all cases. Secondary causes of steatosis including alcohol abuse ($\geqslant$ 140 g/wk), total parenteral nutrition, and the use of drugs known to precipitate steatosis were excluded in all cases. Hepatitis A-G, cytomegalovirus and Epstein-Barr virus infections were ruled out by appropriate tests. In all cases, autoimmune liver disease, metabolic liver disease, Wilson's disease, and α-1-antitrypsin deficiency were ruled out using standard clinical and laboratory evaluation as well as liver biopsy features. Body mass index (BMI), the weight in kilograms divided by the square of the height in meters, was calculated. To compare BMI across different ages and in both boys and girls, BMI Z score was calculated. The Z score represents the number of standard deviations above or below the considered population mean value, based on standardized tables for children[12].

Evaluation of glucose metabolism and insulin sensitivity

All patients underwent a 2-h oral glucose tolerance test (OGTT) with the standard 1.75 g of glucose per kg, or a maximum of 75 g. Glucose tolerance status was determined according to the recently revised American Diabetes Association classification[14] in which a fasting plasma glucose (FPG) level up to 99 mg/dL is considered normal, impaired fasting glucose (IFG) is defined by a FPG of 100-125 mg/dL, impaired glucose tolerance (IGT) is defined by a 2-h plasma glucose of 140-199 mg/dL, diabetes mellitus is defined by a FPG $\geqslant$ 126 mg/dL, or a 2-h plasma glucose $\geqslant$ 200 mg/dL. IFG and IGT are officially termed "pre-diabetes'.

The degree of insulin sensitivity/resistance was determined by the homeostatic model assessment insulin resistence (HOMA-IR) using the formula: IR = (insulin* glucose)/22.5[13], and by the insulin sensitivity index (ISI-comp) derived from OGTT using the formula: ISI = (10 000/square root of [fasting glucose × fasting insulin] × [mean glucose × mean insulin during OGTT])[13]. Both HOMA-IR and the OGTT-derived ISI have a significant correlation with the 'gold standard' euglycemic hyperinsulinemic glucose clamp technique. A HOMA-IR value > 2 and/or ISI-comp value < 6 were considered an indication of insulin resistance.

Liver histology

Biopsies were performed in all children using an automatic core biopsy device (Biopince, Amedic, Sweden) with an 18-G needle (150 mm long), which is able to cut tissue with lengths up to 33 mm with extreme precision[6]. Liver biopsies were at least 15 mm in length and read by a single liver pathologist who was unaware of the patient's clinical and laboratory data. Biopsies were routinely processed (formalin-fixed, paraffin-embedded) and analysed in sections stained with (1) hematoxilin and eosin for overall assessment of parenchymal architecture, hepatocyte abnormalities and inflammatory infiltrates; (2) Van Gieson for assessment of fibrosis and architectural changes; (3) PAS-D after diastase predigestion to highlight debris in portal macrophages and Kupffer cells as well as eosinophilic globules in periportal hepatocytes (characteristic of endoplasmic reticulum storage disease, namely α-1 antitrypsin); and (4) Perl's (Prussian blue) stain for estimation of iron storage in hepatocytes and sinusoidal lining cells. Additionally, immunoistochemical staining with α-1 antitrypsin was used to exclude α-1 antitrypsin-associated liver disease.

The main histological features commonly described in NALFD/NASH including steatosis, inflammation (portal and lobular), hepatocyte ballooning, and fibrosis, were scored according to the scoring system for NAFLD, recently developed and validated by the NIH-sponsored NASH Clinical Research Network[14]. Briefly, steatosis was graded on a 4-point scale: grade 0 = steatosis involving < 5% of hepatocytes; grade 1 = steatosis involving up to 33%of hepatocytes; grade 2 = steatosis involving 33%%-66% of hepatocytes; and grade 3 = steatosis involving > 66% of hepatocytes. Lobular inflammation was graded on a 4-point scale: grade 0 = no foci; grade 1 = less than 2 foci per 200 × field; grade 2 = 2-4 foci per 200 × field; grade 3 = more than 4 foci per 200 × field. Hepatocyte ballooning was graded from 0 to 2: 0 = none, 1 = few balloon cells, 2 = many/prominent balloon cells. Stage of fibrosis was quantified in a 4-point scale: stage 0 = no fibrosis; stage1 = perisinusoidal or periportal fibrosis (1a = mild, zone 3, perisinusoidal; 1b = moderate, zone 3, perisinusoidal; 1c = portal/periportal); stage 2 = perisinusoidal and portal/periportal fibrosis; stage 3 = bridging fibrosis; and stage 4 = cirrhosis. Other features, such as zonal distribution of steatosis, presence of microvesicular steatosis, glicogenated nuclei, lipogranulomas, PAS-D cells, acidophil bodies and Mallory bodies, also were recorded. Portal tract inflammation was graded from 0 to 3 (0 = s none, 1 = mild, 2 = moderate and 3 = severe).

Statistical analysis

Continuous variables were expressed as mean ± SD, while categorical variables were expressed as absolute and percentage frequency. Mann-Whitney rank-sum test and Yates

Table 1 Anthropometric, clinical and biochemical characteristics of patients (n = 69)

Variables characteristics	mean ± SD	Range
Age (mo)	145.7 ± 39.5	44-226
BMI (kg/m^2)	26.3 ± 4.1	15.2-38.3
BMI Z score	2.0 ± 0.79	0.8-5.0
Type II diabetes n (%)	2 (2.3)	
Hypertension n (%)	2 (2.3)	
AST (IU/L)	42 ± 16	19-90
ALT (IU/L)	60 ± 31	10-192
GT (IU/L)	23 ± 20	10-130
Cholesterol (mg/dL)	152 ± 32	75-222
Triglyceride (mg/dL)	86 ± 44.5	28-348
Fasting glucose (mg/dL)	82 ± 11	60-138
Fasting insulin (mU/L)	12.5 ± 6.4	3.5-30.7
HOMA-IR	2.55 ± 1.39	0.67-6.29
ISI-comp	4.4 ± 2.0	1.26-9.16

BMI: body mass index; AST: aspartate aminotransferase; ALT: alanine Aminotransferase; γGT: gamma-glutamyl transpeptidase; HOMA-IR: homeostatic model assessment-insulin resistence; ISI: insulin sensitivity index.

corrected χ^2 were used when appropriate, at the univariate analysis in which several proposed indices for the assessment of fibrosis were assessed with consideration of the following clinical and biochemical parameters, such as glycemia, BMI, body mass index standard deviation score (BMI-SDS), cholesterol, thriglicerides, systolic blood pressure (SBP), dyastolic blood pressure (DBP), ALT, alanine aminotransferase (ALT), AST/ALT ratio, GT, PLT, PT, HOMA, ISI, insulin. The independent role of variables selected by univariate analysis was tested by stepwise logistic regression. Maximal likelihood method was used for entering and removing variables. For all calculations, the biomedical data processing system (BMDP dynamic version 7, University of California, Los Angeles, CA) was used.

RESULTS

Patient characteristics

Clinical details of the 69 children with NAFLD are summarized in Table 1. The median age was 147.5 ± 39.5 (range, 44-226) mo with a male predominance (71%). The mean ALT and AST levels were 60 IU/L (normal values < 36 IU/L) and 42 IU/L (normal values < 32 IU/L) respectively. Insulin resistance, as indicated by a HOMA-IR > 2 or ISI-comp < 6, was present in 37 (53.6%) and 54 (78.2%) children, respectively. There was no significant correlation between BMI Z-score and HOMA-IR (r = 0.15, P = 0.21) or BMI Z-score and ISI-comp (r = 0.13, P = 0.27). The mean total cholesterol and triglyceride levels were 152 mg/dL and 86 mg/dL, respectively. Only 3 children had elevated triglyceride levels (i.e. > 160 mg/dL), and 7 children had hypercholesterolemia (i.e. > 200 mg/dL).

Glucose metabolism

Overall, 67 (97.1%) patients showed normal glucose metabolism on OGTT, whereas the remaining 2 patients (2.9%) showed impaired glucose metabolism, including 1 patient classified as IFG, and 1 patient as IGT. These 2 patients were significantly older (18.4 ± 0.4 *vs* 10.8 ± 4.5 years; P < 0.05) than those with normal glucose metabolism. The BMI Z-score (1.5 ± 1.32 *vs* 1.94 ± 0.67, P = NS) and HOMA-IR (2.35 ± 1.75 *vs* 2.5 ± 1.34, P = NS) were not statistically different between the two groups (no fibrosis *vs* fibrosis).

Table 2 Histological findings in the patients population (Biopsies scored according to Kleiner *et al*), n (%)

Grade/Stage	Steatosis	Necroinflammation	Fibrosis
0	-	12 (17.4)	28 (40.6)
1	25 (36.2)	52 (75.3)	36 (52.2)
2	27 (39.2)	5 (7.2)	1 (1.4)
3	17 (24.6)	-	4 (5.8)
4	-	-	-

Liver histology

The histological findings are summarized in Table 2. All biopsies showed steatosis, mostly macrovesicular. The pattern of steatosis was diffuse or scattered lobular, and only showed zonal distribution in ten cases. Inflammation was present in 57 (82.6%) biopsies. The inflammatory infiltrate was mainly composed of lymphocytes and neutrophils, and when granulomas were present, mononuclear histiocytic cells and eosinophils were also present. Hepatocyte ballooning was present in 34 (49%) of the 69 biopsies, whereas apoptotic cells were noted occasionally. Glycogenated nuclei of variable dimension were present in 35 (50.7%) of the 69 cases, and this nuclear change was noted mostly in zone 1 of the hepatic lobule. No Mallory hyaline was noted in any case, and mild iron deposition was present in 3 cases.

Increased fibrosis was noted in 41 patients (59.4%), mostly of mild (stage 1) severity, one patient in stage 2, and 4 children (5.8%) were showing septal fibrosis (stage 3). Among the 36 patients with stage 1 fibrosis, 3 were 1a, 5 were 1b, and 28 were 1c. No patient showed liver cirrhosis at histology.

Table 3 shows the comparison between children with and without liver fibrosis. Children with liver fibrosis were slightly older, and had significantly higher BMI than those without fibrosis. Those with fibrosis also showed higher serum levels of cholesterol and triglycerides, although the mean values were still within the normal range, and the prevalence of hypercholesterolemia and hypertriglyceridemia was not different between the two groups. Liver enzymes or AST/ALT ratio was not different between those with and without fibrosis.

At multivariate analysis of baseline clinical and biochemical parameters in 28 patients with stage 0 fibrosis in comparison to the 41 patients with stage higher than or equal to 1, only BMI was independently associated with fibrosis (OR = 5.85, 95% IC = 1.6-21). At ROC analysis the cut off value for BMI was 26.3 (sensibility 66%, specificity 71%). Among the 35 patients with BMI ⩾ 26.3, 27 (77.1%) had fibrosis stage ⩾ 1, whereas fibrosis stage ⩾ 1 was present in 14 (41.2%) out of 34 children with BMI < 26.3.

Table 3 Comparison of clinical and biochemical features according to stage of fibrosis (mean ± SD)

Features *n* (%)	No fibrosis 28 (40.6)	Fibrosis ⩾ 1 41 (59.4)	*P*
Age (mo)	140.3 ± 34	151.4 ± 41	0.2
Fasting glucose (mg/dL)	82.4 ± 9.9	81.5 ± 11.7	0.7
BMI (kg/m^2)	25.2 ± 3.3	27.5 ± 4.5	0.01
BMI-SDS	1.84 ± 0.5	2.0 ± 0.7	0.05
Cholesterol (mg/dL)	148.8 ± 34.4	160.2 ± 36.2	0.2
Triglycerides (mg/dL)	82.4 ± 59.5	97.9 ± 39.4	0.2
Systolic pressure (mmHg)	110.2 ± 11.6	116 ± 15	0.07
Diastolic pressure (mmHg)	67.0 ± 8.2	67.1 ± 8.3	0.9
AST (U/L)	41.6 ± 14.6	49.3 ± 32.2	0.18
ALT (U/L)	65.6 ± 26	76.2 ± 82.6	0.4
AST/ALT	0.7 ± 0.3	0.9 ± 0.5	0.05
GT (U/L)	25.2 ± 25	23.7 ± 16.6	0.7
PLT (10^3/L)	285 ± 66	295 ± 59	0.5
PT	87.9 ± 9.3	90.2 ± 7.6	0.3
HOMA-IR	2.7 ± 1.5	2.4 ± 1.1	0.4
ISI	4.0 ± 1.7	4.5 ± 1.9	0.4
Fasting insulin (mU/L)	13.5 ± 6.7	11.6 ± 5.8	0.2

BMI-SDS: body mass index standard deviation score; AST: aspartate aminotransferase; ALT: alanine aminotransferase; PLT: platelets count; PT: prothrombin time; HOMA-IR: homeostatic model assessment-insulin resistence; ISI: insulin sensitivity index.

DISCUSSION

Over the last decades, the prevalence of overweight and obesity among children has increased dramatically, becoming an important public health problem. The negative consequences of paediatric overweight can occur during childhood or adulthood and may result in metabolic, physical, psychosocial and economic consequences. Among these, non alcoholic fatty liver disease has become a growing clinical problem and a major cause of liver-related morbidity.

The accurate means to distinguish between simple fatty liver and steatohepatitis is a liver biopsy as NASH is diagnosed when tissue histology shows fat along with inflammation and damage to liver cells. This distinction has a prognostic relevance because whereas NAFLD is a benign nonprogressive condition, NASH can progress to cirrhosis, liver failure and hepatocellular carcinoma[15]. However, the need for histological evaluation of NAFLD remains controversial as no efficacious treatment strategies have been yet designed. Persistent hypertransaminasemia of non-alcoholic steatohepatitis in obese children may resolve after weight reduction and high doses of vitamin E treatment, but the impact on the natural history needs to be validated by large-cohort controlled studies[16-19]. Thereby, the search for non invasive diagnostic tests of liver fibrotic deposition should be preferred to the expensive and invasive procedure of liver biopsy, whereas the latter should be limited to those cases with uncertain diagnosis[7].

Our previous validation study regarding non invasive diagnosis of liver fibrosis in adults with chronic hepatitis C to cirrhosis showed a significant inverse correlation between stage of fibrosis and platelet count with the highest platelets count in patients with fibrosis 0-2, lower in those with grade 3 and lowest in those with grade 4[20]. Also, an inverse correlation between spleen size and platelets count has been observed (r = - 0.54, $P < 0.0001$) and thrombocytopenia presents in 71% and 23% of patients with or without splenomegaly respectively[21]. The accuracy of platelet count was not significant in children (P = 0.5) as most of the children with NAFLD had stage 1 of fibrosis (52.2%), and less than 8% had a fibrotic score ⩾ 2. The amount of scar deposition that characterizes these stages of fibrosis is too low to favour hemodynamic changes with portal hypertension, altered production of thrombopoietin and enlarged spleen sequestration.

At multivariate analysis of baseline clinical and biochemical parameters in the present study, BMI was an independent predictor of fibrosis (OR = 5.85, 95% CI = 1.6-21); at the cut off value of 26.3 kg/m^2, the BMI evaluation showed a sensibility and specificity of 66% and 71%, respectively, in ascertaining the presence of fibrosis in children. Conceivably, the BMI evaluation may be useful in picking up those children at higher risk of disease progression. Future studies investigating the natural history and the long-term sequelae of our histological findings in children are warranted to corroborate our claim.

In conclusion, increased BMI appears to correlate with long term progression to fibrosis and cirrhosis. Reversal of obesity with a gradual weight reduction can improve laboratory abnormalities, histologic changes and liver size in children with NAFLD. BMI may be considered a good non invasive indicator of the underlying disease. Although little controversy exists about the role of liver biopsy as the best accurate method available to assess the stage of the disease, the decision to perform it should be weighed against the risk of the procedure and the impact of the information obtained. In particular, in paediatric population, the timing of biopsy should be individualized and postponed to non-invasive diagnostic tools.

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COMMENTS

Background
Interest is growing regarding the nonalcoholic steatohepatitis (NASH) in pediatric age and in the usefulness of liver biopsy for its detection.

Research frontiers
Non invasive detection of liver fibrosis may help better define new therapeutical approaches in a larger number of pediatric NASHs.

Innovations and breakthroughs
Still new diagnostic parameters for non invasive detection of liver fibrosis need to be searched and validated in paedriatic population. This work proves that BMI helps identify children with NASH who might have fibrotic deposition in the liver.

Applications
BMI as one of the future criteria can be used for detection of pedriatic patients with NASH-fibrosis.

Peer review
This study tested the accuracy of various clinical and biochemical parameters for the diagnosis of NASH in 69 children. The results show that only increased BMI is significantly associated with fibrosis, whereas other parameters have not been identified as predictive. This study is accurate and is of importance due to the absence of any non invasive method for the diagnosis of liver fibrosis in the paediatric population. While being interesting, this observation should be further confirmed.

S- Editor Wang GP **L- Editor** Wang XL **E- Editor** Liu WF

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World J Gastroenterol 2006 December 28; 12(48): 7826-7831
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Insulin sensitizers in treatment of nonalcoholic fatty liver disease: Systematic review

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Received: 2006-10-10 Accepted: 2006-11-23

Abstract

AIM: To summarize the evidence available for the clinical effectiveness of insulin sensitizers in the treatment of nonalcoholic fatty liver disease (NAFLD) systematically.

METHODS: Relevant articles were located using computer-assisted searches of Medline (1966-March 2006), EMBASE (1988-March 2006), CINAHL (1982-March 2003), Educational Resource Information Center (1966-March 2006), Library, Information Science & Technology Abstracts (1967-March 2006), Cochrane Database of Systematic Reviews, Database of Abstracts of Reviews of Effects (1994-2006), dissertations in ProQuest and FirstSearch databases. Manual searches were made in the abstracts from meetings of the American Gastroenterological Association (1999-2006), and the American Association for the Study of Liver Diseases (2003-2005). Studies were retrieved using the following selection criteria: (1) clinical trials using insulin sensitizers in subjects with NAFLD, (2) adult patients, (3) published as full manuscripts or abstracts, and (4) English, Spanish, German, and French languages only. Data were abstracted independently by two reviewers following standardized procedures. A face-to-face comparison of data was conducted to ensure the completeness and reliability of the abstraction process.

RESULTS: Nine studies were included, six using metformin and three using thiazolidinediones. Only two studies were placebo-controlled trials. The median sample size for all studies was 18 subjects. In the placebo-controlled trials, metformin improved insulin resistance markers and liver function tests, but not histological scores. In the single-arm trials, metformin and thiazolidinediones improved insulin resistance markers and liver function tests, and beneficial histological changes were reported. There is limited high-quality information available from which to draw categorical conclusions about the clinical use of insulin sensitizers in NAFLD.

CONCLUSION: Current information indicates that the use of insulin sensitizers in NAFLD improves insulin resistance and liver function. Histological changes must be corroborated in randomized controlled trials.

Key words: Diet; Metformin; Rosiglitazone; Pioglitazone; Fatty liver; Steatohepatitis; Evidence based medicine; Systematic review

Chavez-Tapia NC, Barrientos-Gutierrez T, Tellez-Ávila FI, Sánchez-Ávila F, Montaño-Reyes MA, Uribe M. Insulin sensitizers in treatment of nonalcoholic fatty liver disease: Systematic review. *World J Gastroenterol* 2006; 12(48): 7826-7831

http://www.wjgnet.com/1007-9327/12/7826.asp

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is an increasingly recognized condition that may progress to end-stage liver disease, ranging from simple steatosis to steatohepatitis, advanced fibrosis, and cirrhosis (in 1.6% of patients with NAFLD). The pathological picture resembles that of alcohol-induced liver injury, but it occurs in patients who do not abuse alcohol[1]. The true prevalence of NAFLD in the USA is unknown. Based on the percentage of people in the Third National Health and Nutrition Examination Survey (NHANES-Ⅲ) with unexplained elevated levels of serum aminotransferase, up to 7.3% of the USA population could be suffering from NAFLD[2]. When the diagnostic criteria are modified, the estimated prevalence of NAFLD reaches 24%[3]. According to

Byron *et al*[4], NAFLD is the third most common diagnosis in gastroenterological referrals, accounting for 11% of patients. NAFLD is expected to become one of the most important liver diseases in the near future as a result of the obesity epidemic[5].

NAFLD was first described more than 20 years ago[1] and many advances in our understanding of its pathophysiological mechanisms have been made. Diet and exercise constitute the central strategies in NAFLD treatment[6]. Considering the pathogenic mechanisms that may be involved, several pharmacological strategies for NAFLD have been tested that focus on correcting the risk factors for insulin resistance and decreasing hyperinsulinemia, as hepatoprotective effects, using diverse drugs: gemfibrozil, metformin, betaine, *N*-acetylcysteine, and vitamin E[7]. However, no consensus regarding an effective therapy for NAFLD has been reached[8].

Over the last five years, clinical trials evaluating the use of insulin sensitizers in the treatment of NAFLD, such as metformin and thiazolidinediones, have been conducted. Mixed results, heterogeneous therapeutic approaches, and the small numbers of subjects have limited their application as clinical guidelines. We performed a comprehensive systematic review to summarize the evidence available for the clinical effectiveness of insulin sensitizers in the treatment of NAFLD.

MATERIALS AND METHODS

Search strategy

Relevant articles were located using computer-assisted searches of Medline (1966-March 2006), EMBASE (1988-March 2006), Cumulative Index to Nursing & Allied Health Literature (CINAHL) (1982-March 2003), Educational Resource Information Center (ERIC) (1966-March 2006), Library, Information Science & Technology Abstracts (LISTA) (1967-March 2006), Cochrane Database of Systematic Reviews (CDSR), Cochrane Controlled Trials Register (CCTR), Database of Abstracts of Reviews of Effects (DARE) (1994-2006), dissertations in ProQuest and FirstSearch databases, and Literatura Latinoamericana y del Caribe en Ciencias de la Salud (LILACS). Manual searches were conducted in the abstracts from the Digestive Disease Week of the American Gastroenterological Association (1999-2006), the American Association for the Study of Liver Diseases Meetings (2003-2005), and the reference lists included in the retrieved articles. Searching terms included: nonalcoholic fatty liver disease, nonalcoholic steatohepatitis, hepatic steatosis, treatment, clinical trial, metformin, thiazolidinediones, rosiglitazone, troglitazone, pioglitazone, englitazone, PPAR-gamma, peroxisomal proliferator activated receptor.

Study selection criteria

Two investigators (ChN, BT) independently reviewed the titles and abstracts of all the citations identified. Potentially relevant studies were retrieved based on the following selection criteria: (1) clinical trials using one or a combination of insulin sensitizers (metformin and thiazolidinediones: rosiglitazone, troglitazone, pioglitazone, englitazone) in subjects with NAFLD, (2) adult patients, (3) data published in full manuscript form or abstract form, and (4) English, Spanish, German, and French languages only.

Eligibility and data abstraction

After retrieval, the articles were subject to evaluation to ensure their compliance with the inclusion and exclusion criteria considered for data abstraction. The exclusion criteria for data abstraction from the selected studies were: (1) use of a concomitant therapeutic approach (ursodeoxycholic acid, antioxidants, *etc*.) with insulin sensitizers, and (2) less than 10 participants at the beginning of the study. Inclusion criteria were: (1) controlled trials of insulin sensitizers versus placebo or diet, (2) single-arm studies of rosiglitazone, troglitazone, pioglitazone, or englitazone, and (3) NAFLD or nonalcoholic steatohepatitis (NASH) based on histological diagnosis or imaging studies (computed tomography, abdominal ultrasound, or magnetic resonance imaging) and/or aberrant liver function tests in the absence of alcohol consumption. Unblinded evaluation of the inclusion/exclusion criteria was conducted separately by all authors. Discrepancies in selection were resolved by consensus. Checks for repeated references were conducted based on the authors' names, publication dates, and reported population characteristics. Data abstraction was conducted independently by ChN and BT following the standardized procedures developed by the research team. The criteria included: publication and study characteristics, study population, diagnostic criteria, intervention description, and baseline and postintervention clinical characteristics. In cases where the information to be abstracted was not presented in the published reports, the authors attempted to contact the corresponding authors (Blaszyk, Azuma, and Duseja), and the responses (Duseja) were included in the abstraction formats. After all studies were abstracted, a face-to-face comparison of data retrieved by ChN and BT was conducted to ensure the completeness and reliability of the abstraction process. Minor discrepancies were recorded and resolved by referring to the original paper.

RESULTS

A total of 94 studies were retrieved from the broad search terms used (Figure 1). After elimination of editorials, reviews, and repeated reports, 10 studies[9-18] remained candidates for evaluation. One study was excluded because of concomitant use of vitamin E[18] (Table 1). Heterogeneity of treatments, methodologies, and reporting quality in the studies precluded any attempt to estimate summary measures, forcing a narrative presentation of our findings. All studies were classified into one of three groups based on the characteristics of the pharmacological interventions: metformin *vs* diet trials, metformin single-arm trials, and thiazolidinedione single-arm trials.

Most (77%) studies were designed as single-arm trials, one was designed as a randomized controlled trial, and one as a nonrandomized controlled trial. Sample sizes ranged

Table 1 Details of trials eligible for this systematic review

Ref.	Design	Participants	Intervention	Outcomes		
				IR	Biochemical	Histological
[9]	Nonrandomized open-label controlled	Italy. 20 consecutive subjects. No diabetic or severely obese subjects.	Treatment: metformin 500 mg/d for 4 mo Control: diet	+	+	NE
[10]	Randomized open-label controlled	Turkey. 36 nondiabetic subjects.	Treatment: metformin 1.7 g/d for 6 mo Control: diet (1600–1800 cal/d)	+	+	–
[11]	Nonrandomized open-label single-arm trial	USA. 15 subjects. One diabetic.	Metformin 20 mg/kg per day for 48 wk	+ at 3 mo + at end of study	+ at 3 mo –(+) at end of study	+[2]
[12]	Nonrandomized open-label single-arm trial	USA. 10 subjects.	Metformin 2 g/d for 48 wk	-	-	-
[13]	Randomized controlled trial[1]	Italy. 17 subjects.	Metformin 2 g/d for 48 wk	+	+	+
[14]	Nonrandomized open-label single-arm trial	India. 22 subjects. Three diabetics.	Metformin 1.5 g/d for 6 mo	+	+	NE
[15]	Nonrandomized open-label single-arm trial	USA. 25 subjects.	Rosiglitazone 8 mg/d for 48 wk plus diet and physical activity	+	+	+
[16]	Nonrandomized open-label single-arm trial	USA. 18 nondiabetic subjects.	Pioglitazone 30 mg/d for 48 wk	+	+	+
[17]	Nonrandomized open-label single-arm trial	Japan. 12 subjects.	Pioglitazone 30 mg/d for 12 wk	+	+	NE

IR: Insulin resistance; NE: Not evaluated. [1]In this study, metformin was compared with vitamin E. We have included only the metformin-treated group, with biopsies at baseline and at the end of the study; [2]Hypothesis test was not provided.

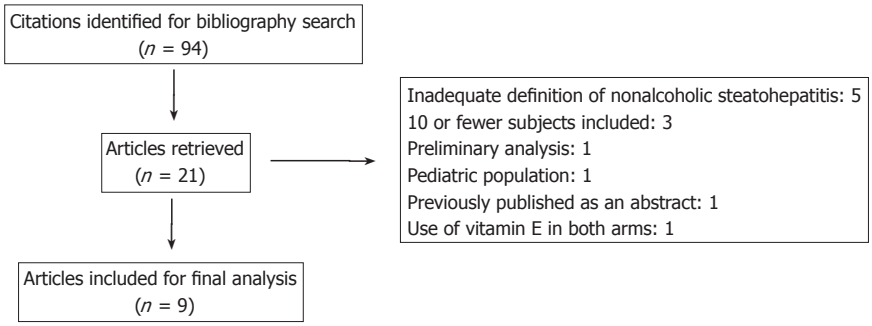

Figure 1 Literature search and selection.

from 10 to 36 subjects, with a median of 18 subjects. All studies used a histological diagnosis of NASH as the inclusion criterion, and posttreatment biopsies were available in only six studies.

Description of the studies

Metformin versus diet trials: Two studies compared the efficacy of metformin versus diet in the treatment of NAFLD. Marchesini *et al*[9] studied 20 consecutive patients (no diabetic or severely obese subjects were included) with liver function tests, and tests for insulin and insulin resistance (by euglycemia and a hyperinsulinemic glucose clamp). Liver biopsies were conducted in 14 subjects who received metformin (500 mg tid) and six were treated with diet alone for four months. The only significant difference between the two groups was in their alanine aminotransferase values. Histological improvement was not evaluated. The diet group did not differ from the drug group in weight reduction, which could reflect the effect of metformin. The most common adverse effect was gastrointestinal. Although subjects undergoing active treatment showed increased levels of lactic acid (by 30% in actively treated patients), just one patient was above the normal range of 2 mmol/L (2.2 mmol/L).

Uygun *et al*[10] studied 36 patients with NAFLD. The treatment group received metformin (850 mg bid) plus dietary treatment. The control group received only a restricted diet (1600-1800 calories per day). Compared with the controls, the treatment group showed improvements in: alanine aminotransferase (37.1 *vs* 17.4 U/L, respectively, $P = 0.003$), aspartate aminotransferase (22.1 *vs* 6.8 U/L, respectively, $P = 0.0001$), body mass

Table 2 Characteristics of single-arm metformin studies

Publication characteristics			Study design				Intervention			Diagnosis	Demographics			
First author	Year	Publication type	Study design	Randomization	Blinding	Sample size	Drug	Doses reported	Treatment duration	NASH	Male (%)	Age (yr)	Obese (%)	Diabetics (%)
Nair[11]	2004	Article	Open label	N	N	15	Metformin	20 mg/kg/d	48 wk	Histology	53	51	NR	6
Blaszyk[12]	2005	Abstract	Open label	N	N	70	Metformin	2 g/d	48 wk	Histology	70	NR	NR	NR
Bugianesi[13]	2005	Article	RCT[1]	Y	N	17	Metformin	2 g/d	48 wk	Histology	83	44	22–38	0
Duseja[14]	2006	Article	Open label	N	N	17	Metformin	1500 mg/d	24 wk	Histology	68	36	NR	13

Author	AST (U/L)			ALT (U/L)			Insulin resistance				Biopsies		NASH score			Adverse effects			
	Initial	Final	*P*	Initial	Final	*P*	Method	Initial	Final	*P*	Initial (*n*)	Final (*n*)	Initial	Final	*P*	Diarrhea	Lactate increase	Dropout rate	Loss to follow-up
Nair	47	41	NS	62	68	NS	QUICKI	0.306	0.315	< 0.05	15	10	1.4	1	NR	15	6	1	0
Blaszyk	NR	NR	NR	109	82	NR	HOMA	11.4	5	NR	10	10	2.4	2.1	NS	NR	NR	0	0
Bugianesi	44	25	< 0.05	96	36	< 0.05	HOMA	5.8	2.8	0.0006	17	17	6.5	4.4	< 0.001	NR	1	0	0
Duseja	NR	NR	NR	124.6	74.4	NR	KITT	1.42	2.03	NS	22	0	12	NA	NA	0	0	0	0

[1]In this study, metformin was compared with vitamin E. We have included only the metformin-treated group, with biopsies at baseline and at the end of the study. RCT: Randomized controlled trial; NR: Not reported, NS: Not significant; NA: Not applied; QUICKI: Quantitative insulin sensitivity check index; HOMA: Homeostatic model assessment; KITT: K index of insulin tolerance test.

Table 3 Characteristics of single-arm thiazolidinediones studies

Publication characteristics			Study design				Intervention			Diagnosis	Demographics			
First author	Year	Publication type	Study design	Randomization	Blinding	Sample size	Drug	Doses reported	Treatment duration	NASH	Male (%)	Age (yr)	Obese (%)	Diabetics (%)
Azuma[17]	2002	Abstract	Pilot study	N	N	12[1]	Pioglitazone	15 mg/d	12 wk	Histology	66	40	NR	0
Neuschwander-Tetri[15]	2003	Article	Open label	N	N	30	Rosiglitazone	8 mg/d	48 wk	Histology	46	45	13	6
Promrat[16]	2004	Article	Pilot study	N	N	18	Pioglitazone	30 mg/d	48 wk	Histology	39	45	61	11

Author	AST (U/L)			ALT (U/L)			Insulin resistance				Biopsies		NASH score			Adverse effects			
	Initial	Final	*P*	Initial	Final	*P*	Method	Initial	Final	*P*	Initial (*n*)	Final (*n*)	Initial	Final	*P*	Weight gain	Anemia	Dropout rates	Loss to follow-up
Azuma	NR	NR	NA	110	39	< 0.05	Insulin	54	30	< 0.05	12	0	NR	NR	NA	NR	NR	5	0
Neuschwander-Tetri	60	34	< 0.05	89	41	< 0.05	HOMA	7.5	4	< 0.05	26	22	NR	NR	NA	20	[2]	5	0
Promrat	61	34	< 0.05	99	40	< 0.05	HOMA	4.3	2.6	< 0.05	18	18	NR	NR	< 0.05	13	0	1	0

[1]Only seven patients in the treatment group. Information about the control group is not available; [2]This study reported anemia as an adverse effect, but numerical data are not available. NR: not reported; NA: not applied; HOMA: homeostatic model assessment.

index (2.4 *vs* 1.9 kg/m^2, respectively, $P = 0.01$), and index of insulin resistance (1.15% *vs* 0.02%, respectively, $P = 0.001$). In fact, a comparison of the treatment group at baseline and at six months showed improvements in alanine aminotransferase (83.5 ± 24.6 *vs* 46.4 ± 23.3 U/L, respectively, $P = 0.0001$) and aspartate aminotransferase (57.9 ± 17.3 *vs* 35.8 ± 10.5 U/L, respectively, $P = 0.0001$). However, this was also seen in the control group: alanine aminotransferase (72.8 ± 31.2 *vs* 55.4 ± 16.3 U/L, respectively, $P = 0.001$) and aspartate aminotransferase (48.1 ± 26.3 *vs* 41.3 ± 13.5 U/L, respectively, $P = 0.06$). No differences were observed in the liver biopsies of subjects after treatment. No patient discontinued metformin because of a lack of tolerance for the treatment. No patient reported symptoms of hypoglycemia. Four patients complained of gas and bloating and two patients complained of mild to moderate abdominal pain in the first month. However, these complaints did not require cessation of the drug.

Metformin single-arm trials: Four single-arm trials evaluated the use of metformin in NAFLD (Table 2)[11-14]. The mean age of the study participants ranged from 36 to 51 years in all but one study[14]. Males were predominant (ranging from 53% to 83%). The doses used in the different series ranged from 20 mg/kg per day (approximately 1.4 g per day in a subject of 70 kg) to 2 g/d. Treatment duration varied from six months[14] to 48 wk. Insulin resistance was assessed by the QUICKI, HOMA, or KITT methods.

All trials reported an improvement in the indices of insulin resistance, three studies[12-14] reported a reduction in liver function test values, and one study reported a nonsignificant increase in these values[11].

In terms of histological improvement, only one report[13] showed statistical differences in inflammation, steatosis, fibrosis, and global evaluations of NASH after treatment. The most common adverse effects were associated with poor gastrointestinal tolerance. One patient had an increase in serum lactate levels that required the patient to withdraw from the study.

Thiazolidinediones single-arm trials: The use of thiazolidinediones (pioglitazone, rosiglitazone, and

troglitazone) was evaluated in three studies (Table 3)[15-17]. The mean age in each study was 40-46 years. In one study, men were in the majority[17], and in two studies, diabetic subjects were included[15,16]. Of these studies, two used pioglitazone and one used rosiglitazone, at varying doses (pioglitazone 15-30 mg/d and rosiglitazone 4 mg, bid). The durations of the studies ranged from 12 wk[17] (pioglitazone 15 mg/d) to 48 wk. Two studies assessed insulin resistance with HOMA-IR[15,16] and the other with serum insulin levels[17]. Posttreatment hepatic biopsies were reported in two studies[15,16].

All studies showed significant improvement in insulin resistance. Alanine aminotransferase and aspartate aminotransferase showed significant decreases in all studies. Posttreatment biopsies showed statistically significant improvements with respect to baseline biopsies[15,16]. The adverse effects reported were weight gain, serum lactate increases, bad dreams, and heavy legs. The pooled dropout rate was high, at 11 of 60 subjects. No cases of liver failure were reported.

DISCUSSION

This systematic review analyzes the clinical use of insulin sensitizers in the treatment of NAFLD. Although it has been more than 20 years since the first description of NAFLD[1], and much progress has been made in understanding its epidemiology and pathophysiology, few advances have been made in its treatment.

In this review, only two clinical studies compared pharmacological treatments with diet treatments. The methodological limitations are clear: the small numbers of subjects, nonrandomization and the lack of blinded measures, and the limited use of histological outcomes.

The fact that limited high-quality information available is interesting, especially because (1) NAFLD is a very common disease, with incidences between 3% in the low-risk population[19] and 93% in high-risk subjects[20,21], (2) subjects have some degree of histologically evident chronic liver damage, and at least 30% have fibrosis at diagnosis[22], (3) it is an important cause of chronic liver failure and adversely affects survival rates, with 7-10-year liver-related mortality rates of 12% to 25%[23], and (4) it is an important factor in cardiovascular-related mortality; in a 10-year prospective study of subjects with NASH or hepatitis C viral infection, the mortality rates were 5.2% *vs* 0.6%, respectively ($P < 0.03$)[24].

Analyzing the usefulness of insulin sensitizers by comparing metformin with thiazolidinediones in single-arm trials suggests that thiazolidinediones are the better option. However, when diet-controlled studies are considered, this conclusion is less clear because, contrary to the single-arm trials, these studies indicate that the use of metformin clearly benefits liver enzymes. Unfortunately, no data from a well-designed head-to-head comparative clinical trial are available to answer this question. In all the studies analyzed, a heterogeneity of drugs and doses was observed, which made it more difficult to evaluate the efficacy of insulin sensitizers in clinical practice.

The evidence presented in this systematic review indicates that the treatment of NAFLD with insulin sensitizers has been, until now, a nebulous field. However, new well-designed trials have been in progress during the preparation of this paper. Four trials using metformin and three studies on thiazolidinediones are in the recruitment phase[25]. Information derived from these studies should help in the clinical management of this disease.

Despite this (and future) information, many issues are not answered: (1) cost-analysis comparing diet and exercise with pharmacological treatment, (2) safety of insulin sensitizers in large samples, and perhaps one of the most important questions that (3) insulin sensitizers only treat one face of the metabolic syndrome and pharmacological approaches to treat all components of the metabolic syndrome sounds too simplistic[26]. This indicates that more creative prevention policy is mandatory.

In conclusion, current information indicates that the use of insulin sensitizers in the treatment of NAFLD improves insulin resistance and liver function. Single-arm studies have shown positive histological changes. However, placebo-controlled trials do not support this histological response. Future information derived from well-designed running trials will be useful in defining the clinical implications of insulin sensitizers in the treatment of NAFLD.

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S- Editor Liu Y **L- Editor** Zhu LH **E- Editor** Bai SH

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World J Gastroenterol 2006 December 28; 12(48): 7832-7836
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Diagnosis and treatment of gallbladder perforation

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Received: 2006-09-06 Accepted: 2006-11-07

Abstract

AIM: To present our clinical experience with gallbladder perforation cases.

METHODS: Records of 332 patients who received medical and/or surgical treatment with the diagnosis of acute cholecystitis in our clinic between 1997 and 2006 were reviewed retrospectively. Sixteen (4.8%) of those patients had gallbladder perforation. The parameters including age, gender, time from the onset of symptoms to the time of surgery, diagnostic procedures, surgical treatment, morbidity, and mortality were evaluated.

RESULTS: Seven patients had type Ⅰ gallbladder perforation, 7 type Ⅱ gallbladder perforation, and 2 type Ⅲ gallbladder perforation according to Niemeier's classification. The patients underwent surgery after administration of intravenous electrolyte solutions, and were treated with analgesics and antibiotics within the first 36 h (mean 9 h) after admission. Two patients died of sepsis and multiple organ failure in the early postoperative period. Subhepatic abscess, pelvic abscess, pneumonia, pancreatitis, and acute renal failure were found in 6 patients.

CONCLUSION: Early diagnosis and emergency surgical treatment of gallbladder perforation are of crucial importance. Upper abdominal computerized tomography for acute cholecystitis patients may contribute to the preoperative diagnosis of gallbladder perforation.

Key words: Acute cholecystitis; Gallbladder perforation; Early diagnosis; Computed tomography; Emergency surgery

Derici H, Kara C, Bozdag AD, Nazli O, Tansug T, Akca E. Diagnosis and treatment of gallbladder perforation. *World J Gastroenterol* 2006; 12(48): 7832-7836

http://www.wjgnet.com/1007-9327/12/7832.asp

INTRODUCTION

Gallbladder perforation (GBP) is a rare but life threatening complication of acute cholecystitis. Sometimes GBP may not be different from uncomplicated acute cholecystitis with high morbidity and mortality rates because of delay in diagnosis[1-3]. Thus GBP still continues to be an important problem for the surgeons. Most cases can only be diagnosed during surgery[1,4]. Male acute cholecystitis cases with high fever, high white blood cell (WBC) count, and associated systemic diseases should be meticulously investigated[1,5,6].

Niemeier[7] in 1934, classified free gallbladder perforation and generalized biliary peritonitis as acute or type Ⅰ GBP, pericholecystic abscess and localized peritonitis as subacute or type Ⅱ GBP, and cholecystoenteric fistula as chronic or type Ⅲ GBP. This classification is still in use. We aimed to present our clinical experience with GBP in this study.

MATERIALS AND METHODS

Subjects

Records of 332 patients who received medical and/or surgical treatment with the diagnosis acute cholecystitis in our clinic between January 1997 and February 2006 were reviewed retrospectively. Sixteen (4.8%) of those patients were found to have gallbladder perforation. Perforations due to trauma, iatrogenic causes, and gallbladder (GB) carcinoma were excluded.

Methods

The original classification of Niemeier[7] was used to identify the patients. The parameters including age, gender, time from the onset of symptoms to the time of surgery, diagnostic procedures, surgical treatment, postoperative morbidity and mortality were evaluated. Direct abdominal X-ray series, abdominal ultrasound scanning (US), abdominal contrast-enhanced computerized tomography (CT), routine blood cell count, and blood chemistry tests were performed. Peritoneal spaces were lavaged thoroughly with isotonic saline and drains were placed for postoperative drainage in all of the patients.

RESULTS

There were 10 male and 6 female patients. Their mean age was 69 (range, 54-85) years. Their complaints were abdominal pain, poor general condition, high fever, nausea, and vomiting on admission. Cholelithiasis was unknown prior to perforation in 5 patients. The patients

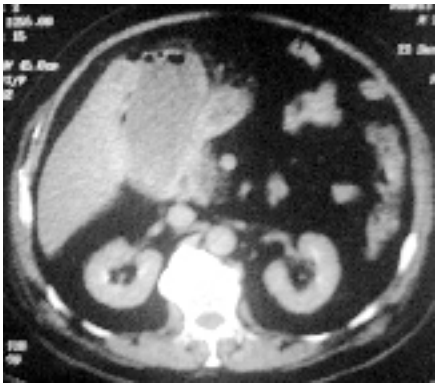

Figure 1 Contrast-enhanced CT image at the level of gallbladder.

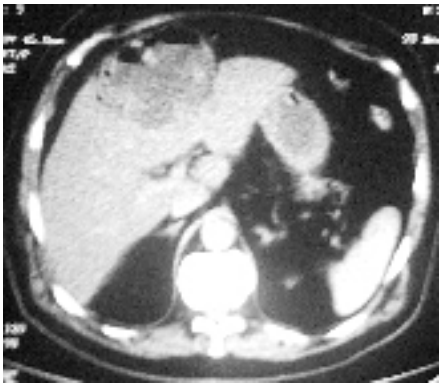

Figure 2 Contrast-enhanced CT image of hepatic abscess adjacent to the superior part of gallbladder.

had several associated diseases, of which atherosclerotic heart disease (AHD) was the most common. One patient with type I gallbladder perforation was on long term steroid treatment for systemic lupus erythematosus. White blood cell (WBC) count was high in 14 patients, and 10 patients had high fever. Patients with type I gallbladder perforation had signs of peritoneal irritation such as extensive abdominal tenderness, guarding and rebound tenderness. Patients with type Ⅱ gallbladder perforation had local tenderness, guarding, positive Murphy's sign and 4 of them had palpable right subcostal mass while one patient had jaundice with a total bilirubin level of 28 mg/dL. Two patients with type Ⅲ gallbladder perforation had epigastric tenderness and one of them also had abdominal distension. Four patients had systemic inflammatory response syndrome, of them two patients with type I gallbladder perforation and two with type Ⅱ and type Ⅲ gallbladder perforations. The number of patients and their clinical features in each type of gallbladder perforation are shown in Table 1.

Abdominal X-ray series and abdominal US were performed for all of the patients and abdominal CT scanning for 14 patients. Only one patient with type Ⅲ gallbladder perforation had air-fluid levels on direct abdominal radiograms. Abdominal US showed gall stones in all of the patients with type I and type Ⅱ gallbladder perforations, extensive intraperitoneal free fluid in 7 patients with type I gallbladder perforation, and a small amount of pericholecystic free fluid in 6 patients with type Ⅱ gallbladder perforation. Abdominal US did not show GB wall defect in any of the patients. CT revealed GB wall thickening in all of the patients, gall stones in 10 patients, extensive intraperitoneal free fluid in 5 patients, a small amount of pericholecystic free fluid in 7 patients, and GB perforation sites in 5 patients. Abdominal CT and US detected liver abscesses in 2 patients, dilated extra and intrahepatic bile ducts in 1 patient. Abdominal CT showed dilated intestinal loops suggesting mechanical obstruction in one patient with type Ⅲ gallbladder perforation. Abdominal CT showed dilated stomach suggesting gastric outlet syndrome in another patient. The stomach was dilated with a normal mucosa, but the gall stone could not be seen during upper gastrointestinal endoscopy of this patient.

Table 1 Clinical features of the patients, *n* (%)

Feature	Type Ⅰ	Type Ⅱ	Type Ⅲ
n (%)	7 (43.7)	7 (43.7)	2 (12.6)
Mean age (yr)	67.5	68.4	76.5
Gender (Male/Female)	5/2	5/2	0/2
Fever (more than 38℃), *n* (%)	5 (71.4)	5 (71.4)	-
White blood cell count (mean)	23966	17314	13200
Mean duration of symptoms (d)	5	8.6	15
Systemic disorders[1], *n* (%)			
Atherosclerotic heart disease	4 (14.3)	4 (57.1)	1 (50)
Diabetes mellitus	2 (28.6)	1 (14.3)	1 (50)
Hypertansion	1 (14.3)	1 (14.3)	1 (50)
Systemic lupus erythematosus	1 (14.3)	-	-
Chronic obstructive pulmonary disease	1 (14.3)	2 (28.6)	1 (50)
Systemic inflammatory response syndrome	2 (28.6)	1 (14.3)	1 (50)
Preoperative diagnoses, *n* (%)			
Gallbladder perforation	4 (14.3)	1 (14.3)	-
Acute cholecystitis	2 (28.6)	4 (14.3)	-
Cholangitis	-	1 (14.3)	-
Peptic ulcus perforation	1 (14.3)	-	-
Perforated appendicitis	-	1 (14.3)	-
Mechanical bowel obstruction	-	-	1 (50)
Gastric outlet syndrome	-	-	1 (50)
Sites of perforation, *n* (%)			
Fundus	6 (85.7)	3 (42.9)	-
Corpus	1 (14.3)	2 (28.6)	-
Infundibulum	-	1 (14.3)	2 (100)
Cystic duct	-	1 (14.3)	-

[1]Some patients had more than one sytemic disorder.

Enlarged gallbladder, irregularity of the gallbladder walls at the fundic region, air density lateral to the fundus and corpus, and increased density of the mesentery anterior to the fundus suggesting free fluid on CT image of a patient with type Ⅱ gallbladder perforation are shown in Figure 1. The abdominal CT image of a patient with hepatic abscess adjacent to the superior part of GB is shown in Figure 2.

The patients underwent surgery after administration of intravenous crystalloid solutions, and were treated with analgesics and antibiotic (third generation cephalo-

sporins) within the first 36 h (mean 9 h) after admission. The patients who had associated diseases such as diabetes, chronic obstructive pulmonary disease (COPD), and AHD underwent surgery after specific medical treatment was started. Antibiotic treatment was changed when required, according to the results of microbiological assessment of the infected bile specimens obtained from the gall bladder. The most common preoperative diagnoses were perforated cholecystitis in patients with type I gallbladder perforations, and acute cholecystitis in patients with type Ⅱ gallbladder perforations. Laparoscopic cholecystectomy was performed in 6 patients, two of them had type I gallbladder perforation, and four type Ⅱ gallbladder perforation. Conversion was required in 2 of them due to unclear anatomy. Laparotomy was performed in the remaining 10 patients. Perforations were recognized intraoperatively in 11 patients. The perforated site of the GB was the fundus in 9 patients, the corpus in 3 patients, the infundibulum in 3 patients, and the cystic duct in 1 patient. Two patients with type Ⅱ gallbladder perforation also had hepatic abscesses, which were covered by the omentum. Hepatic abscesses were also drained in these patients. All of the patients received a cholecystectomy. Common bile duct exploration was performed, gall stones were extracted, and a T-tube was placed into the common bile duct in addition to cholecystectomy in 1 patient with obstructive jaundice. One of the 2 patients had a cholecystoduodenal fistula (type Ⅲ gallbladder perforations) underwent surgery for gastrointestinal tract obstruction due to bile stones in the jejunum, which were removed through an enterotomy. Gall stones were also found in the duodenum of the other patient with type Ⅲ gallbladder perforation and removed through a duodenotomy.

The median hospital stay was 15 d (4-26 d). Two patients (12.5%) died of sepsis and multiple organ failure in the early postoperative period. One was a female patient at the age of 79 with type Ⅲ gallbladder perforation and COPD. The other was a 84 year-old male patient with delayed type Ⅱ gallbladder perforation and pericholecystic abscess associated with AHD and diabetes. Subhepatic abscess, pelvic abscess, pneumonia, pancreatitis and acute renal failure were found in 6 patients (Table 2). Subhepatic and pelvic abscesses were drained percutaneously under ultrasound guidance. The other diseases were treated conservatively.

Table 2 Surgical procedures and morbidities of the patients *n* (%)

	Type Ⅰ	Type Ⅱ	Type Ⅲ
Surgery			
Laparoscopic cholecystectomy	2 (12.5)	4 (25)	-
Laparotomy and cholecystectomy	5 (31.2)	3 (18.7)	2 (12.5)
Morbidity[1]			
Subhepatic abscess		1 (11.1)	1 (11.1)
Pelvic abscess		1 (11.1)	1 (11.1)
Pneumonia	1 (11.1)	1 (11.1)	1 (11.1)
Pancreatitis		1 (11.1)	
Acute renal failure	1 (11.1)		

[1]Some patients had more than one morbidity.

DISCUSSION

Inflammation may progress and cause ischemia and necrosis, thus resulting GBP in 2% to 11% of acute cholecystitis patients[6,8,9]. GBP also develops following acalculous cholecystitis, although rare[10,11]. GB fundus, the most distal part with regard to blood supply, is the most common site of perforation[8,12]. The incidence of GBP was 4.5% and the most frequent site of perforation was the fundus (60%) in our study. Six of the 7 type I gallbladder perforations and 3 of 7 type Ⅱ gallbladder perforations were at the fundus. When GB is perforated at the fundus, it is less possibly covered by the omentum, thus the bile drains into the peritoneal space. If the perforation site is not at the fundus, it is easily sealed by the omentum or the intestines and the condition remains limited in the right upper quadrant with formation of a plastrone and pericholecystic fluid. This observation suggests that if the perforation site is at the fundus, it is more likely to end up with a type I perforation. The relation between the site and the type of GBP has not been defined. Although statistical analysis was not possible because of the insufficient number of patients in this series, this observation may be supported by larger series.

Acute uncomplicated cholecystitis is more common among females with a female to male ratio of 2:1[13]. However, GBP is more frequent in male gender[1,6]. In our study, male patients made up of 60% and the two patients with type Ⅲ gallbladder perforation were females. GBP is usually seen over 60 years of age[4,14]. Roslyn *et al*[1] reported that type I and Ⅱ GBP tend to occur in younger patients, especially more or less at the age of 50 years, whereas type Ⅲ gallbladder perforations are more common in the elderly. The patients with type I gallbladder perforation were relatively younger than those with type Ⅱ and Ⅲ gallbladder perforations in our study.

Type I gallbladder perforations are usually seen in patients with AHD, diabetes, malignancy, cirrhosis, and immunosupressive diseases, or during immunosupressive treatment, without a history of chronic cholecystitis. On the other hand, type Ⅲ gallbladder perforations most often occur in patients with a previous long time history of gall stones[1,9,12]. Severe AHD has been reported in 21% of patients with type I and Ⅱ gallbladder perforations, and diabetes, in 25% of patients with type I gallbladder perforation[8,14]. Such high rates are related to vascular disorders caused by these systemic diseases. It has been reported that type Ⅱ GBP occurs more frequently[1,3,9,12]. The incidence of type I and Ⅱ gallbladder perforations was equal and the most frequent associated systemic disease was AHD in the present study. Type Ⅲ gallbladder perforations usually occur in chronic cholecystitis patients with obstructive gastrointestinal symptoms[9,12,15]. Since the symptoms of type Ⅰ-Ⅱ GBP and uncomplicated cholecystitis are similar, differential diagnosis may be difficult based on physical examination, laboratory tests, and radiological methods and the diagnosis may not be established

preoperatively[6,12]. Delay in diagnosis is the major cause of its high morbidity and mortality[1,4,9,16]. Tanaka *et al*[17] reported that only one patient had a concrete diagnosis preoperatively in their series of 9 patients. The majority of GBP patients include those who undergo early surgery with the diagnosis of acute cholecystitis. The main complaint of the patients is abdominal pain accompanied with nausea and vomiting during the last 7 d[12,18]. The duration of symptoms was shorter in patients with type Ⅰ gallbladder perforation than in patients with type Ⅱ and Ⅲ gallbladder perforation in our study. High fever and elevated WBC count are not the diagnostic indications for gallbladder perforation. Parker *et al*[19] reported that high fever and high WBC count could be observed in 56% and 59% of patients with acute cholecystitis, respectively. The majority of type Ⅰ and Ⅱ gallbladder perforation cases had fever whereas type Ⅲ gallbladder perforation cases did not in our study. The patients with type Ⅰ and Ⅱ gallbladder perforation had elevated WBC count, but those with type Ⅲ gallbladder perforation had only a mild increase in WBC count.

US findings in acute cholecystitis, such as the GB wall thickening, GB distension, pericholecystic free fluid, and positive sonographic Murphy sign, may also be present in gallbladder perforation cases[2,9,20]. Sood *et al*[2] noted that the sonographic hole sign, in which the defect in GB wall is visualized, is the only reliable sign of gallbladder perforation. They reported that GB wall defect could be shown with a high resolution ultrasound scanner device in 70% of patients[2]. However, Kim *et al*[21] reported that the site of defect could not be visualized on US in any patients, which is similar to our study. On the other hand, CT can show more accurate signs of free intraperitoneal fluid, pericholecystic fluid, and abscess[1,2,22]. CT can also show GB wall thickness and the defect on the wall due to perforation[2,20]. In our study, all of the 5 patients who had the diagnosis of gallbladder perforation preoperatively were diagnosed by CT. Since the patients were admitted for acute abdominal pain, standard pelvic CT rather than upper abdominal CT was applied. Kim *et al*[21] reported that the defect could not be visualized on CT in 54% of patients. Doppler ultrasound, magnetic resonance imaging and radionuclide methods have been used in the diagnosis of gallbladder perforation[23-25]. The majority of our patients with type Ⅱ gallbladder perforation were initially treated conservatively and then underwent surgery as no improvement was observed during the first 3 d.

Cholecystectomy, drainage of abscess if present, and abdominal lavage are usually sufficient to treat gallbladder perforation[1,4]. Cholecystectomy may be difficult in type Ⅲ gallbladder perforations. If a cholesystectomy is performed, additional surgical procedures such as repair of the fistula may be required[15,16]. Cholecystectomy can be performed after the infection is relived by US guided percutaneus drainage in type Ⅱ gallbladder perforations[22]. Laparoscopic cholecystectomy can be performed for acute, gangrenous, and/or perforated cholecystitis as well as uncomplicated cholecystitis, but a conversion may be necessary in case of difficulties like an unclear anatomy[9,16]. In our study, laparoscopic procedure was initiated in 6 patients but conversion was required in two.

Since the difficulties in diagnosis cause delay in treatment, higher morbidity and mortality rates are often encountered[1,2,9]. Glenn and Moore[26] have reported that the mortality rate of gallbladder perforation patients is 42%, while other studies reported that the mortality rates are decreased to 12%-16% owing to the developments in anesthesiology and intensive care conditions[1,3]. The morbidity and mortality rates were 37.5% and 12.5%, respectively in the present study.

In conclusion, early diagnosis of gallbladder perforation and immediate surgical intervention are of crucial importance. Although standard abdominal CT has an important role in diagnosing gallbladder perforation, upper abdominal CT for acute cholecystitis in which pericholecystic fluid is found by US may increase the rate of preoperative diagnosis of gallbladder perforation.

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S- Editor Wang GP **L- Editor** Wang XL **E- Editor** Bai SH

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World J Gastroenterol 2006 December 28; 12(48): 7837-7843
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

A head to head comparison of oral *vs* intravenous omeprazole for patients with bleeding peptic ulcers with a clean base, flat spots and adherent clots

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Received: 2006-09-29 Accepted: 2006-11-22

Yılmaz Ş, Bayan K, Tüzün Y, Dursun M, Canoruç F. A head to head comparison of oral *vs* intravenous omeprazole for patients with bleeding peptic ulcers with a clean base, flat spots and adherent clots. *World J Gastroenterol* 2006; 12(48): 7837-7843

http://www.wjgnet.com/1007-9327/12/7837.asp

Abstract

AIM: To compare the effect of intravenous and oral omeprazole in patients with bleeding peptic ulcers without high-risk stigmata.

METHODS: This randomized study included 211 patients [112 receiving *iv* omeprazole protocol (Group 1), 99 receiving *po* omeprazole 40 mg every 12 h (Group 2)] with a mean age of 52.7. In 144 patients the ulcers showed a clean base, and in 46 the ulcers showed flat spots and in 21 old adherent clots. The endpoints were re-bleeding, surgery, hospital stay, blood transfusion and death. After discharge, re-bleeding and death were re-evaluated within 30 d.

RESULTS: The study groups were similar with respect to baseline characteristics. Re-bleeding was recorded in 5 patients of Group 1 and in 4 patients of Group 2 (P = 0.879). Three patients in Group 1 and 2 in Group 2 underwent surgery (P = 0.773). The mean length of hospital stay was 4.6 ± 1.6 d in Group 1 *vs* 4.5 ± 2.6 d in Group 2 (P = 0.710); the mean amounts of blood transfusion were 1.9 ± 1.1 units in Group 1 *vs* 2.1 ± 1.7 units in Group 2 (P = 0.350). Four patients, two in each group died (P = 0.981). After discharge, a new bleeding occurred in 2 patients of Group 1 and in 1 patient of Group 2, and one patient from Group 1 died.

CONCLUSION: We demonstrate that the effect of oral omeprazole is as effective as intravenous therapy in terms of re-bleeding, surgery, transfusion requirements, hospitalization and mortality in patients with bleeding ulcers with low risk stigmata. These patients can be treated effectively with oral omeprazole.

Key words: Oral omeprazole; Peptic ulcer; Bleeding

INTRODUCTION

Acute upper gastrointestinal (UGI) bleeding remains the most common reason for emergency hospital admission managed by gastroenterologists. It is reported that it has an annual incidence ranging from approximately 50 to 150 per 100 000 of the population[1], and it is still rising steadily in the aspirin/nonsteroidal antiinflammatory drugs (NSAIDs) era. The most common cause of UGI bleeding in adult patients is peptic ulcer disease, which accounts for about 50% of the cases[2]. Patients with peptic ulcer bleeding account for an overall mortality rate that has remained around 5%-10% for the past five decades, despite improved therapy options and the availability of intensive care units[3].

Previous consensus guidelines and several studies have demonstrated that the risk for re-bleeding or continued bleeding from an ulcer is strongly associated with the stigmata seen at endoscopic examinations[4-7]. These hemorrhagic stigmata consist of a clean ulcer base, flat spots, adherent clots, nonbleeding visible vessels and active bleeding (oozing and spurting). Major stigmata of recent hemorrhage include spurting, oozing vessels, nonbleeding visible vessels or fresh adherent clots, while an old adherent clot was considered as minor stigmata[8]. There are also several studies that classified the high-risk stigmata as spurting, oozing, or nonbleeding visible vessels, excluding all adherent clots[9,10]. Currently, endoscopic hemostatic therapy is strongly recommended in patients with arterial spurting, oozing ulcers and nonbleeding visible vessels[11,12]. The optimum management of adherent clots has long been controversial. Although there are studies with opposite conclusions in this field[13,14], a cited meta-analysis showed that endoscopic therapy is of significant benefit in patients with active bleeding or a visible vessel but not in patients with adherent clots[15].

The overuse of parenteral proton pump inhibitors (PPI) in UGI bleeding is a common practice all over the world.

A recent meta-analysis pointed out that both intravenous (*iv*) and oral (*po*) PPIs are effective in UGI bleeding. However, the mortality is increased with *iv* PPI in the same report[16]. Moreover, it is reported that most patients who present with ulcer bleeding have low-risk stigmata and do not require *iv* PPI treatment but can be appropriately and adequately treated with oral PPIs in clinical practice[17]. In case intravenous treatment is particularly expensive, oral treatment would be appropriate.

Most previous studies on omeprazole have been performed with *iv* administration and not with *po* form. It is known that most physicians do not prefer *iv* PPI in patients with low-risk ulcers. In spite of this view, the issue needs a better confirmation. In the literature there are an abundant number of studies that compare oral PPI *vs* placebo, with[18] or without[19] endoscopic therapy, *iv* PPI *vs* placebo[20] or oral PPI *vs* endoscopic injection[11]. At the same time, to the best of our knowledge, there is no study in the literature that has been designed to allow head-to-head comparison of oral *vs* intravenous PPI treatment in UGI bleeding. We therefore designed this study to make a comparison of oral and intravenous omeprazole in patients with bleeding peptic ulcers without high-risk stigmata, in terms of re-bleeding, surgery, hospital stay, blood transfusion and mortality. We believe that it is important to add stronger study-supported evidence to the literature in this population.

MATERIALS AND METHODS

Patients, definitions and study design

Patients were enrolled in the study if they had any symptoms of upper gastrointestinal bleeding, such as hematemesis, melena or the presence of blood in a patient' s nasogastric tube lavage. They were all older than 18 years. Informed consent to participate in the study was obtained from all patients and the study was performed in accordance with the principles stated in the Declaration of Helsinki. Exclusion criteria were as follows: (1) a history of chronic liver disease and portal hypertension, (2) gastroduodenal malignancy, (3) gastric surgery, (4) known adverse drug reactions to the trial drugs, (5) current use of antisecretory drugs, H_2-receptor antagonists or PPIs, (6) a history of endoscopic therapy for bleeding ulcer within the past four weeks, (7) pregnancy or lactation, (8) had endoscopic findings of active bleeding (spurting, oozing vessels or nonbleeding visible vessels), (9) refusal to provide written informed consent. Moreover, patients found to have malignant ulcers after initial enrolment were also excluded.

Gender, age, current smoking, alcohol consumption, comorbid medical illnesses, use of aspirin/NSAIDs (any dose within last week), prior epigastric pain, history of previous upper gastrointestinal bleeding, prior major surgery, concomitant use of anticoagulants, antithrombocytic agents other than aspirin, and steroids and previous eradication treatment for *H pylori,* were investigated. The spectra of the comorbid illnesses included chronic obstructive pulmonary disease, pneumonia, end-stage renal disease with hemodialysis, chronic renal insufficiency or acute renal failure, congestive heart failure, coronary artery disease and cerebrovascular accident. Besides, we recorded the duration of hospitalization, number of re-bleeding episodes, initial hemoglobin level, coagulation parameters, need for blood transfusion, the endoscopic data and addresses/phone numbers of all patients.

Endoscopic examinations were performed using a videoendoscope (Olympus GIF-V70, Tokyo, Japan) within the first 24 h of admission. At endoscopy all primary and secondary lesions were recorded. The coagulation factors (prothrombin time, partial thromboplastin time, platelet count) were checked and corrected prior to any endoscopic intervention, if needed. Patients with an underlying anatomic cardiac abnormality were considered at a high risk for endocarditis, and recommended antibiotic regimens were given. An ulcer was defined as a circumscribed mucosal break at least 5 mm in diameter and with a perceptible depth. The ulcer size was measured using biopsy forceps, of which the fully opened cup was 5 mm in diameter. Besides, stigmata of recent hemorrhage were recorded. A 'fresh' adherent clot was defined as the presence of an adherent clot over the ulcer that could not be dislodged by vigorous washing with a jet of water delivered through the channel of the endoscope[21]. An 'old' clot was defined, on the contrary, as a clot dislodged easily by washing. These lesions were also excluded from the study due to their needs for an endoscopic therapy. All patients with benign gastroduodenal ulcers showing a clean ulcer base, flat spots or old adherent clots at endoscopy were included in the study. During the emergency admission, oral anticoagulant therapy was stopped in users and coagulation was corrected when applicable. The criteria for blood transfusion were as follows: hemoglobin levels of lower than 9 g/dL in older than 65 years, hemoglobin levels of lower than 8 g/dL in younger patients, or if the patient had a new episode of hematemesis in both age groups. Besides, if a state of shock existed, blood was transfused independent of haemoglobin levels. All patients with upper gastrointestinal bleeding were examined for *H pylori* in biopsy specimens taken from the antrum by hematoxylin and eosin (HE) staining.

We performed a single-center randomized clinical trial, comparing the effect of high dose intravenous omeprazole (Group 1) and oral omeprazole (Group 2) on bleeding peptic ulcer. The study was conducted between January 2004 and August 2006 at Gastroenterology Clinic of Dicle University Research Hospital in Turkey. After a stabilization period, patients were randomly divided into two groups in the endoscopy laboratory. A person outside from the study staff placed the two drug formulations into sealed non-transparent envelops and coded them based on random table numbers. Only this person knew the codes. The research assistant, other medical personnel, the endoscopists, and patients were blind to this information. The study was conducted in a double-blind manner as all treatment assignments were revealed at the end of the study. The high dose intravenous group received a bolus injection of omeprazole (Losec®, AstraZeneca, Molndal, Sweden), 80 mg, given at admission, followed immediately by a continuous infusion of 8 mg/h for 72 h, then 40

mg orally daily for 6 wk. The other group received oral omeprazole (Omeprol®, Ilsan-Hexal (Sandoz), Gebze, Turkey) 80 mg a day (20 mg capsule, two in the morning and two in the evening) for 72 h, then 40 mg orally daily for 6 wk. It is well-known that PPI treatment is an essential option in bleeding peptic ulcers and we did not include a placebo group for each treatment due to ethical problems.

The primary endpoints of the study were recurrent bleeding (early re-bleeding), surgery requirement, and death rates before discharge. Re-bleeding was defined as new hematemesis, melaena, or hypotension (< 100 mm Hg systolic blood pressure) associated with a drop in haemoglobin and/or endoscopic evidence of fresh re-bleeding. Patients with recurrent bleeding underwent urgent second endoscopy for confirmation and the lesion was classified as in previous description. Surgical intervention was considered if the bleeding could not be controlled by endoscopic therapy. Shock was defined as a pulse rate > 100 beats/min, systolic blood pressure < 100 mmHg accompanied by cold sweats, pallor, and oliguria. Secondary endpoints were duration of hospital stay, blood transfusion requirement, and re-bleeding or death within 1 mo after index bleeding (late re-bleeding). All patients were also evaluated in terms of risk analysis by Rockall scoring system, which is based on five variables (age, presence of shock, comorbidity, endoscopic diagnosis, and endoscopic stigmata)[22].

Follow-up

Each patient was visited in the ward daily by a clinical research assistant who recorded information about their condition, their management, and results. After the treatment procedures, we observed the patients for complications such as recurrent bleeding, perforation or death in the hospital. Blood pressure and pulse rate were monitored hourly during the first 24 h and every 4 h, hemoglobin levels every 4 h during the first day and daily thereafter until discharge. Those who had no evidence of recurrent bleeding were discharged as soon as possible. After a treatment protocol of 72 h, patients with histologically proven *H pylori* were prescribed a 2-wk course of full dose omeprazole, twice daily, amoxicillin 1 g twice daily and clarithromycin 500 mg twice daily, irrespective of the treatment protocol. In addition, we recommended them not to use aspirin/NSAIDs if not needed anymore or use them in combination with PPIs. After discharge, all of the patients were informed about our contact phone number and the patients or their relatives were asked to report to us if any re-bleeding or death occurred within 30 d.

Statistical analysis

Data were entered into a personal computer and analysed using the Epi-INFO 2000 software package (version 2000, CDC, Atlanta). Continuous variables were presented as mean (standard deviation). The results of the two treatment groups were compared by χ^2 test, Student's *t* test and Fisher's exact tests in the analysis as appropriate. To test the association between outcomes and clinical covariables, we estimated risk ratios and 95% CI. In all analyses, statistical significance was defined as $P < 0.05$.

RESULTS

During the study period, a total of 278 patients with bleeding gastroduodenal ulcers were admitted to our clinic. Of these ulcers, 21 were actively bleeding, 17 had nonbleeding visible vessels and 17 had a fresh adherent clot. At the beginning, all these 55 patients, together with 5 patients with malignant ulcer presentation, 4 patients currently known to take antisecretory drugs, H_2-RAs or PPIs and 3 patients with gastric surgery were excluded from the study.

Thus, a total of 211 eligible patients were included in the study and all of them completed the treatment protocols. The mean age of the patients was 52.7 (range, 18-93 years). The total number of patients with duodenal ulcer was 160 (75.8%) and gastric ulcer 51 (24.2%). Of the ulcers, 144 (68.2%) had a clean base, 46 (21.8%) had flat spots and 21 (10.0%) had old adherent clots. There were 112 patients in Group 1 (taking *iv* omeprazole) and 99 patients in Group2 (taking *po* omeprazole). The study groups were similar with respect to gender, age, stigmata of ulcer hemorrhage, use of aspirin/NSAIDs, *H pylori* status and previous eradication treatment, co-existing illnesses, previous abdominal surgery, alcohol consumption, smoking habit, previous epigastric pain, previous UGI bleeding, hematemesis, coagulopathy, shock, hematocrit, ulcer site (gastric-duodenal) and size. The characteristics of patients in both groups are summarized in Table 1. Multiple ulcers were found to be more common in Group 2 compared to Group 1 (6 and 17, respectively, $P = 0.007$). Besides, gastric antral and corporal ulcers were more common in Group 2 compared to Group 1 (33 *vs* 18, respectively, $P = 0.007$), while the number of duodenal anterior and posterior ulcers was similar in both groups. *H pylori* infection was present in 61.2% patients with duodenal ulcer and 41.2% patients with gastric ulcer ($P = 0.012$). Aspirin/NSAID use was recorded in 82% of gastric ulcers, while in 62% of duodenal ulcers ($P = 0.007$).

Clinical outcomes during hospital stay (inpatient basis)

Recovery without major complications was seen in 107 (95.5%) patients of Group 1 and in 95 (96.0%) patients of Group 2 ($P = 0.945$). Recurrent bleeding was recorded in 5 (4.5%) patients assigned to Group 1 and 4 (4.0%) patients assigned to Group 2 ($P = 0.879$, Fisher's exact test). Re-bleeding rates were similar between duodenal and gastric ulcers, and posterior duodenal and gastric corporal ulcers ($P = 0.511$ and 0.673, respectively. Fisher's exact test). Only one ulcer with a clean base (11.1%), while 3 ulcers with old clots (33.3%) and 5 with flat spots (55.5%) showed rebleeding.

Three patients (2.7%) in Group 1 and 2 (2.0%) in Group 2 underwent surgery to control re-bleeding after a failure in second endoscopic intervention ($P = 0.773$, Fisher's exact test). Surgery requirement was mostly seen in patients taking aspirin/NSAIDs in both groups (2, for each). Four patients (1.9%), two in each group died ($P = 0.981$, Fisher's exact test). Three were older than 65 years. The Rockall score higher than 8 was present in 18.1% of Group 1 and 19.1% of Group 2. The causes of death were pneumonia in 2, myocardial infarction in 1 and pneumonia

Table 1 Baseline characteristics of the study groups

Characteristics	Group 1 (*iv*) (*n* = 112)	Group 2 (*po*) (*n* = 99)	Total (*n* = 211)	*P*
Male / Female (*n*)	79/33	66/33	145/66	0.545
Age (mean ± SD)	52.7 ± 17.05	52.8 ± 19.61	52.7 ± 18.12	0.966[1]
Age < 65 yr [*n* (%)]	80 (71.4)	64 (64.6)	144 (68.2)	0.291
Age ⩾ 65 yr [*n* (%)]	32 (28.6)	35 (35.4)	67 (31.8)	
Ulcer site (*n*):				
-Duodenal	94	76	160	0.083
-Gastric	18	23	51	
Endoscopic signs (*n*):				
-Clean base	82	62	144	0.244
-Flat spot	20	26	46	
-Old adherent clot	10	11	21	
Ulcer size (cm)	1.05 ± 0.4	1.06 ± 0.6	1.05 ± 0.5	0.934[1]
Ulcer count (*n*):				
-Single	106	82	188	0.007[2]
-Multiple	6	17	23	
Ulcer locations (*n*):				
-Posterior duodenal	61	52	113	
-Anterior duodenal	33	14	47	0.007
-Gastric corporal	9	16	25	
-Gastric antral	9	17	26	
Aspirin/NSAIDs use [*n* (%)]	68 (60.7)	70 (70.7)	138 (65.4)	0.072
H pylori positive [*n* (%)]	63 (56.3)	56 (56.6)	119 (56.4)	0.963
Previous eradication [*n* (%)]	10 (8.9)	3 (3.0)	13 (6.2)	0.09[2]
Coexisting illness [*n* (%)]	41 (36.6)	35 (35.4)	76 (36.1)	
-Cardiac (*n*)	18	14	32	0.850
-Pulmonary (*n*)	16	15	31	
-Cerebral (*n*)	7	6	13	
Previous surgery [*n* (%)]	19 (17.0)	22 (22.2)	41 (19.4)	0.335
Alcohol [*n* (%)]	5 (4.5)	2 (2.0)	7 (3.3)	0.452[2]
Smoking [*n* (%)]	47 (42.0)	38 (38.4)	85 (40.3)	0.597
Previous pain [*n* (%)]	72 (64.3)	67 (67.7)	139 (65.9)	0.604
Previous bleeding [*n* (%)]	17 (15.2)	17 (17.2)	34 (26.1)	0.694
Hematemesis [*n* (%)]	77 (68.8)	69 (69.7)	146 (69.2)	0.882
Coagulopathy [*n* (%)]	4 (3.5)	3 (3.0)	7 (3.3)	0.917[2]
Hematocrit (%, Mean)	24.2 ± 3.2	23.6 ± 3.4	23.9 ± 3.1	0.567[1]
Index hematocrit < 25% (*n*)	47	45	92	0.610
Index hematocrit ⩾ 25% (*n*)	65	54	119	
Shock (*n*)	6	5	11	0.381[2]
Rockall score ⩽ 3 [*n* (%)]	63 (56.3)	52 (52.5)	115 (54.5)	0.737
> 8 [*n* (%)]	21 (18.7)	19 (19.1)	40 (18.9)	0.865

[1]Student's *t* test; [2]Fisher's exact test; NSAIDs: non-steroidal anti-inflammatory drugs.

Table 2 Clinical outcomes of the study population

Outcome	Group 1 (*iv*) (*n* = 112)	Group 2 (*po*) (*n* = 99)	Total (*n* = 211)	*P*
Inpatient basis				
Recovery [*n* (%)]	107 (95.5)	95 (96.0)	202 (95.7)	0.945
Re-bleeding [*n* (%)]	5 (4.5)	4 (4.0)	9 (4.3)	0.879[2]
Surgery requirement [*n* (%)]	3 (2.7)	2 (2.0)	5 (2.4)	0.773[2]
Hospital stay (days, mean)				
Total	4.6 ± 1.6	4.5 ± 2.6	4.5 ± 2.8	0.710[2]
⩽ 5 d (*n*)	52	55	107	0.093
> 5 d (*n*)	60	44	104	
Blood transfusion (units)				
Total (mean)	1.9 ± 1.1	2.1 ± 1.7	2.0 ± 1.6	0.350[1]
⩽ 3 units (*n*)	85	74	159	0.847
> 3 units (*n*)	27	25	52	0.610
Death [*n* (%)]	2 (1.8)	2 (2.0)	4 (1.9)	0.981[2]
Outpatient basis				
Re-bleeding [*n* (%)]	2 (1.8)	1 (1.0)	3 (1.4)	0.766[2]
Death [*n* (%)]	1 (0.8)	0 (0.0)	1 (0.4)	0.887[2]
Overall				
Re-bleeding [*n* (%)]	7 (6.2)	5 (5.0)	12 (5.6%)	0.745[2]
Death [*n* (%)]	3 (2.6)	2 (2.0)	5 (2.3)	0.980[2]

[1]Student's *t* test; [2]Fisher's exact test.

Table 3 Probable effects of variables on re-bleeding

Factor	Re-bleeding (*n*)	*P*	OR (95% CI)
Co-existing illness	5	0.288	2.3 (0.6-8.8)
Hematemesis	6	0.971	0.8 (0.2-3.6)
Smoking	5	0.490	1.9 (0.4-7.3)
Aspirin/NSAIDs	7	0.721	1.1 (0.3-8.7)
Age ⩾ 65 yr	5	0.117	2.1 (0.7-10.8)
Ulcer size > 1 cm	6	0.001	11 (2.6-46.3)
Ulcer locations:			
Posterior duodenal	4	0.041	7 (1.5-18.2)
Gastric corporal	3	0.049	5 (1.0-14.3)
Ulcer stigmata:			
Flat spots	5	0.001	12 (4.5-57.3)
Old clots	3	0.023	5 (1.2-13.5)

plus adrenal insufficiency in 1. Total hospital stay was 3 d at minimum and 20 d at maximum. The mean duration of hospital stay was 4.6 ± 1.6 d in Group 1 and 4.5 ± 2.6 d in Group 2. Length of hospital stay did not differ significantly between two groups ($P = 0.710$, Student's *t* test). Hospital stay more than 5 d was also similar between the groups ($P = 0.093$). The median number of units of blood transfused was approximately 2 in each group ($P = 0.350$, Student's *t* test). Blood transfusion requirement was more than 3 units in 27 (24.1%) patients of Group 1 and 25 (25.3%) patients of Group 2 ($P = 0.610$). Details about clinical outcomes are summarized in Table 2.

Bleeding from posterior duodenal (44.4%) and gastric corporal (33.3%) sites was more common compared to other sites [$P = 0.041$, OR 7 (1.5-18.2) and $P = 0.049$, OR 5 (1.0-14.3), 95% CI], respectively]. Of ulcers with re-bleeding, a diameter greater than 1 cm had a higher risk [$P = 0.001$, OR 11.0 (2.6-46.3), 95% CI]. Ulcers with flat spots and old clots had also higher risks for re-bleeding. Co-existing illnesses, hematemesis, smoking habit, aspirin/NSAIDs use and age older than 65 years did not have any effect on re-bleeding rates. The risk estimates of cofactors and their powers are summarized in Table 3.

Clinical outcomes after discharge in 30 d (outpatient basis)

We strictly informed patients or their relatives that it was very important to report to us any problems (new bleeding attack or death) immediately that occurred during the discharge period. None of the patients took aspirin or NSAIDs during the 30 d follow-up period. Four patients (or a relative) re-contacted us within 30 d. A new bleeding occurred in 2 patients of Group 1 and in 1 patient of

Group 2 after the index bleeding episode. The overall re-bleeding was seen in 12 (5.6%) patients [7 (6.2%) in Group 1 and 5 (5.0%) in Group 2]. Additionally, one patient died from Group 1 due to a new cerebrovascular event. Hence, the overall death was seen in 5 (2.3%) patients.

DISCUSSION

In the present study, we demonstrated that oral omeprazole was as effective as intravenous omeprazole in controlling bleeding peptic ulcers without high-risk stigmata. Importantly, the study implies that treatment with the oral agent is indicated for the bleeding instead of the *iv* approach when in reality, PPI use in this situation is simply to heal the ulcer. Although most of bleeding episodes from peptic ulcers resolve spontaneously and are not detrimental, recurrence of bleeding adversely affects prognosis. The overall re-bleeding rate in the oral treatment group (5.0%) was similar to that in the intravenous treatment group (6.2%) within 30 d, and both groups were also similar with respect to the need for surgery, duration of hospitalization, total amounts of blood transfusion, and mortality. Although clean base ulcers form the largest portion of the study population, the calculated Rockall scores were higher than 8 in approximately one fifth of the patients. As well-known, a score of higher than 8 is associated with a high risk of death[22]. Comorbidity and age were the predominant contributors to these high scores in our population.

Although the Federal Drug Administration has not approved intravenous proton pump inhibitors for the treatment of UGI bleeding, these agents are being used widely all around the world. A meta-analysis pointed out that PPI therapy in UGI bleeding was effective only in patients with UGI bleeding caused by peptic ulcers and with high-risk stigmata for re-bleeding[16]. Moreover, it has been reported that patients with endoscopy results showing a low risk of re-bleeding should not be treated endoscopically as their prognosis is excellent when treated conservatively[1].

What about oral versus intravenous drug administration? Most of the previous studies on omeprazole have been conducted with intravenous omeprazole and not with the oral drug. The oral absorption of omeprazole is 50%; however, as gastric pH rises, as much as 75% may be absorbed. It is highly protein-bound in plasma and is rapidly metabolized in the liver, and the metabolites are excreted in urine. The onset of antisecretory effect occurs within 1 h, with peak effects occurring in 2 h, depending on the dose[23]. Demonstration of effectiveness of oral treatment would be particularly attractive as it would allow treatment to be initiated outside, prior to hospital admission. However, it was concluded that pharmacotherapy alone could not replace endoscopic hemostasis for patients with actively bleeding ulcers or ulcers with nonbleeding visible vessels[12]. On the other hand, it was reported that oral omeprazole therapy can be a valid alternative to endoscopic therapy, especially when injection therapy is not readily available[11]. Replacement of endoscopy in bleeding may be more possible in ulcers with low risk stigmata. Interestingly, one meta-analysis warned readers that those patients with UGI bleeding with significant comorbid diseases (such as diabetes mellitus, collagen vascular disease and hypercoagulable states) need careful monitoring, as intravenous PPI therapy in such patients may be harmful. Besides, all-cause deaths and non-ulcer deaths in trials using intravenous PPI were higher in the treatment group and not in trials using oral PPI[16].

The role of oral omeprazole in bleeding peptic ulcers was studied, especially in ulcers with high-risk stigmata by some authors and they found the rebleeding rates of omeprazole groups to be 7%, 10.9%, 15%, 22.9% and 26%, respectively[11,18,19,24,25]. Bour *et al*[25], Khuroo *et al*[19] and Jung *et al*[11] did not perform endoscopic therapy in omeprazole groups, while Javid *et al*[24] and Kaviani *et al*[18] did so. Jung *et al*[11] reported that oral omeprazole administration was comparable to endoscopic ethanol injection therapy for prevention of re-bleeding in patients with nonbleeding visible vessels or adherent clots. Moreover, Kaviani *et al*[18] showed that oral omeprazole reduced the re-bleeding rate, hospital stay and need for blood transfusion even in high-risk ulcers after endoscopic therapy. Khuroo *et al* reported a lower re-bleeding rate with oral omeprazole alone compared to placebo[19], which is comparable to the re-bleeding rates achieved by endoscopic therapy alone[13,14]. Bour *et al* also reported that oral omeprazole was comparable to endoscopic injection therapy[25], but they administered a smaller dose of omeprazole (40 mg every 24 h) than we did. This dosage may not be sufficient to maintain intragastric pH > 4. Detailed studies *in vitro* have shown that at a pH of < 6, the extrinsic and intrinsic coagulation cascades are impaired, and platelet aggregation is virtually abolished[26]. In our study, omeprazole at a dose of 40 mg every 12 h was administered because the gastric pH was reported to be > 6 during 85% of the first 24 h period at that dose[19].

Although it was declared four years ago by the British Society of Gastroenterology Endoscopy Committee[1] that patients who have active bleeding from the ulcer, a non-bleeding visible vessel, or have adherent clots should be recommended to receive endoscopic therapy (grade A), it was subjected to strong objections[27] and the disapproval was supported by a meta-analysis[15]. After a clot has been diagnosed, approaches to its management are quite different. Removal of blood clots is probably more hazardous in centers where clinicians are less experienced in handling peptic ulcer bleeding. However, targeted irrigation has been shown repeatedly to be safe and should be widely adopted in managing ulcers with adherent clots[28]. Randomized, controlled trials of endoscopic therapy versus non endoscopic therapy for ulcers with adherent clots have yielded conflicting results[13,29], and a meta-analysis does not support routine use of endoscopic therapy[15]. Our study groups had a low count (10 *vs* 11) of adherent clots and they had a lower risk for re-bleeding. The risk for re-bleeding with clots that remained adherent after washing has been reported to be only 8%[30]. We repeated the analysis excluding clots and found out that re-bleeding rates were also similar in both groups. On the other hand, the rebleeding rate of 10.8% (5/46) in the flat spot group is higher compared with other studies[4]. Interobserver variability of stigmata classification could be

a major limitation in this condition.

H pylori infection and chronic aspirin/NSAID use are the two major risk factors among patients hospitalized for ulcer bleeding[17]. Eradication of *H pylori* has been demonstrated in many randomized, controlled trials[31,32], to reduce the rate of ulcer recurrences and rebleeding in complicated ulcer diseases. In a recent study, duodenal ulcers were more likely to be associated with *H pylori* infection than gastric ulcers. In contrast, gastric ulcers were more likely associated with aspirin/NSAID use than were duodenal ulcers[17]. In patients with upper gastrointestinal bleeding, the sensitivity of the rapid urease test is relatively low in detecting *H pylori*. It was reported that this was best accomplished by histologic examination with a sensitivity above 90%[33], and therefore we did so. In our study, *H pylori* infection was present in 98 (61.2%) patients with duodenal ulcer, and in 21 (41.2%) patients with gastric ulcer ($P = 0.012$). Aspirin/NSAID use was seen in 82% of gastric ulcers, while in 62% of duodenal ulcers ($P = 0.007$). It was also reported that prior use of aspirin/NSAIDs increases the risk of re-bleeding in bleeding ulcer patients, and leads to a higher need for urgent surgery[34]. In parallel with this conclusion, surgery requirement was mostly seen in patients taking aspirin/NSAIDs in both groups (2, for each) in our study. On the other hand, endoscopic features of high-risk included ulcer size (> 1 or 2 cm)[34,35] and the site of bleeding (the posterior lesser gastric curvature and posterior duodenal wall)[36-38]. Bleeding from posterior duodenal (44.4%) and gastric corporal (33.3%) ulcers was more common compared to other sites in our study (Table 3). The mean age of our study population was 52 years, and more than one third had co-existing illnesses.

In conclusion, our results suggest that the effectiveness of oral omeprazole administration is comparable to intravenous therapy in terms of re-bleeding, need for emergency surgery, transfusion requirements, length of hospital stay and mortality in patients with bleeding peptic ulcers without high risk stigmata. In most of the countries, most patients with bleeding ulcers have low risk stigmata, and thus, can be treated with oral omeprazole. These patients do not explicitly require expensive omeprazole infusions.

ACKNOWLEDGMENTS

The authors are thankful to Sandoz Pharmaceutical Company for their contribution in providing capsules for patients who could not obtain the drug. The authors would also like to thank the nursing staff (Şehnaz Cingöz and Yılmaz Beyaztaş) of the Endoscopy Center of Dicle University Hospital for their generous support.

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COMMENTS

Background

As yet, there is no study in the literature that had a head-to-head comparison of oral *vs* intravenous proton pump inhibitor treatment in bleeding peptic ulcers. We designed a study to compare the effect of *iv* and *po* omeprazole in patients with bleeding peptic ulcers without high-risk stigmata.

Research frontiers

We demonstrate that the effect of oral omeprazole is as effective as intravenous therapy in terms of re-bleeding, surgery, transfusion requirements, hospitalization and mortality in patients with bleeding ulcers with low risk stigmata. These patients can be treated effectively with oral omeprazole.

Innovations and breakthroughs

Our results suggest that the effectiveness of oral omeprazole administration is comparable to intravenous therapy in terms of re-bleeding, need for emergency surgery, transfusion requirements, length of hospital stay and mortality in patients with bleeding peptic ulcers without high risk stigmata. Generally speaking, most patients with ulcer bleeding have low-risk stigmata, and thus, can be treated with oral omeprazole. These patients do not explicitly require expensive omeprazole infusions.

Peer review

It is a practical research to compare the effectiveness of oral omeprazole *vs iv* omeprazole in peptic bleeding cases without high risk stigmata. The results showed that oral treatment is as effective as the iv treatment but less expensive. The study was well designed with enough material as well as statistical analysis.

S- Editor Liu Y **L- Editor** Zhu LH **E- Editor** Liu WF

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World J Gastroenterol 2006 December 28; 12(48): 7844-7847
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Depression in patients with irritable bowel syndrome in Jos, Nigeria

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Received: 2006-07-20 Accepted: 2006-11-27

Abstract

AIM: To study the brain-gut interaction and the effect of behavioral or psychiatric conditions on irritable bowel syndrome (IBS) in an African population.

METHODS: IBS was diagnosed using the Rome Ⅱ diagnostic criteria. The entry of each patient was confirmed following detailed explanations of the questions. Four hundred and eighteen patients were studied. Subjects satisfying the Rome Ⅱ criteria for IBS were physically examined and stool microscopy was done to identify the presence of "alarm factors". Depression was diagnosed using the symptom-check list adapted from the Research Diagnostic Criteria (DSM-Ⅳ) of the American Psychiatric Association.

RESULTS: Seventy-five (56.8%) of the 132 IBS patients were depressed whereas only 54 (20.1%) of the 268 non-IBS patients were depressed. There was a significant relationship between IBS and depression (χ^2 = 54.29, Odds ratio = 5.21, 56.8 ± 8.4 *vs* 20.1 ± 5.2, *P* = 0.001). Even though constipation predominant IBS patients were more likely to be depressed, no significant relationship was found between the subtype of IBS and depression (χ^2 = 0.02, OR = 0.95, *P* = 0.68).

CONCLUSION: IBS is significantly associated with major depression but not gender and bowel subtypes of the patients. Patients with IBS need to be evaluated for depression due to the highly significant relationship between the two conditions.

Key words: Irritable bowel syndrome; Depression; Nigeria; Africa

Ladep NG, Obindo TJ, Audu MD, Okeke EN, Malu AO. Depression in patients with irritable bowel syndrome in Jos, Nigeria. *World J Gastroenterol* 2006; 12(48): 7844-7847

http://www.wjgnet.com/1007-9327/12/7844.asp

INTRODUCTION

Irritable bowel syndrome (IBS) has been widely studied in the western world and pathophysiologic mechanisms have been available to explain the constellation of symptoms[1]. IBS is a chronic disorder of unknown etiology clinically consisting of altered bowel habits, abdominal pain and the absence of any detectable organic pathologic process[1]. Along with the putative pathophysiologic mechanisms of post-infectious inflammatory disorder, disordered intestinal motility and visceral hypersensitivity, there has been much discussion regarding the brain-gut interaction and the influence of behavioral or psychiatric conditions on these symptoms[2].

IBS is recognized widely as one of the most commonly encountered gastrointestinal disorders[1-4]. Locally, there is paucity of data concerning IBS in Africa. Of the studies available however, IBS was reported in 30 percent of an African population at the University College Hospital, Ibadan[5] and 8% in Kenya[6]. Peak prevalence of IBS was reported in the third decade of life in the study in Kenya. A more recent report from our centre demonstrated a prevalence of 26.1% among a healthy student population[7]. In addition, patients with IBS are at increased risk for other non-gastrointestinal functional disorders such as fibromyalgia[8-11] and interstitial cystitis[12]. IBS is known to co-exist with some psychiatric disorders such as depression, anxiety disorders, somatoform disorders[9,13] and sexual dysfunction (e.g., dyspareunia)[14]. The situation is however, not known in African population. This study aims at determining the prevalence of depression among African IBS patients.

MATERIALS AND METHODS

Patients

This is a descriptive cross-sectional study. The principal investigator administered the questionnaire after obtaining informed consent to randomly selected subjects attending the General Outpatient (GOP) Clinics of the three hospitals in Jos. These hospitals were: Jos University Teaching Hospital (JUTH), Evangel Hospital (EEH) and

Plateau State Specialist Hospital (PSSH). The GOP clinics of these hospitals are where primary care physicians attend to patients prior to referrals for specialist care. On selected days of the week, the investigators went to the various clinics until the proposed study sample size was achieved.

The number of patients visiting each of the GOP clinics in the previous year was used to divide the total sample to be studied proportionately. This was obtained from the records of the hospitals. Individual patients were randomly selected in each GOP clinic. Every fifth patient at EEH, every fourth patient at JUTH and alternate patient at PSSH were selected, subject to satisfying the inclusion criteria.

The study was approved by the Ethics Committee of the three hospitals prior to the commencement of the study. The study at EEH was conducted between July 2003 and September 2003, PSSH between October and November 2003, and JUTH between December 2003 and February 2004. Each patient's entry was confirmed following detailed explanations of the questions. Subjects satisfying the Rome Ⅱ criteria for IBS were physically examined to identify the presence of "alarm factors." Major depression was diagnosed using the symptom-check list adapted from the Research Diagnostic Criteria (DSM-Ⅳ) of the American Psychiatric Association[15].

Sample size

The minimum sample size was determined based on the prevalence reported in the literature using the following formula:

$$N = \frac{(Zi\text{-}a)^2 (P) (1\text{-}P)}{d^2}$$

N = minimum sample size; P = best estimate of prevalence of IBS from the literature review expressed as a fraction of 100 (in this case, 30% = 0.3)[5]; Zi-a = a constant at 95% confidence interval for a two-tailed descriptive study (= 1.96); d = absolute precision, i.e. value required (in percentage) which in actual terms describes the maximum difference between the population rate and the sample rate that can be tolerated. Five percent (0.05) was adapted for this study. Thus:

$$N = \frac{1.96^2 (0.3) (0.7)}{(0.05)^2} = 323$$

However, 400 subjects were recruited for the study. Systematically selected adult patients aged 18 to 50 years attending the GOP clinics of the three hospitals were included and the pregnant women and subjects using laxatives for any reason, those with memory problems and the presence of alarm symptoms, including a positive stool test for helminthes were excluded from the study.

The 400 patients who attended the three selected hospitals and who satisfied the inclusion criteria were studied with the questionnaires by the principal investigator. Four hundred and eighteen patients were initially evaluated, but 18 were excluded for various reasons. Six were above 50 years of age, 4 had significant weight loss, 5 could not give a coherent history, 2 declined to participate in the study and 1 had recurrent bloody stools. Of the 400 subjects, 174 were from ECWA Evangel Hospital, 148 from Jos University Teaching Hospital, and 78 from Plateau State Specialist Hospital.

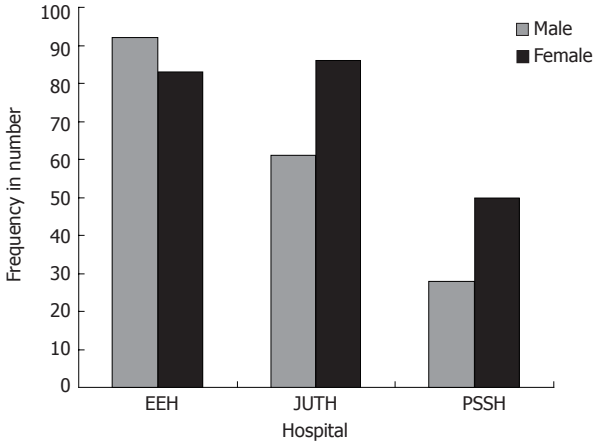

Figure 1 Distribution of study population by gender in the three GOP clinics.

Statistical analysis

Data were analysed using the Epi Info 2000 Statistical Program[16]. Means and standard deviations were used to describe continuous variables and proportions for categorical data. Two-tailed Student's *t* test and analysis of variance (ANOVA), a parametric test for inequality of population means, were used for comparison of group means while the significance of observed differences (proportions) was determined by Chi-square test. $P < 0.05$ was considered statistically significant.

RESULTS

The mean age of the study population at EEH, JUTH and PSSH were 32.5 ± 9.3, 32.0 ± 10.0 and 31.1 ± 8.8 years, respectively and with an age range of 18-50 years at each hospital. There was no statistically significant difference in the mean age among the three study populations (f = 0.62, $P = 0.54$). Figure 1 shows the gender distribution of the study population in the three GOP clinics. Eighty-three (47.4%) females and 92 (52.6%) males were recruited at the EEH, while 86 (58.5%) females and 61 (41.5%) males were recruited at JUTH. Fifty (64.1%) females and 28 (35.9%) males were selected at PSSH.

Age and gender distribution of study subjects

The mean age of this population was 32.0 ± 9.4 years with a range of 18-50 years. The mean age for females was 31.9 ± 9.5 years and males 32.1 ± 9.3 years, showing no statistically significant difference ($t = 0.2$, $P = 0.8$). Subjects in the third decade of life predominated over the other age groups, and the number of females was higher than their male counterparts.

Irritable bowel syndrome and depression

Table 1 gives a summary of this relationship. Seventy-five (56.8%) of the 132 IBS patients were depressed whereas only 54 (20.1%) of the 268 non-IBS patients were depressed. There was a significant relationship between IBS and depression (Odds ratio = 5.21, $\chi^2 = 54.29$, $P = 0.001$). There was no significant relationship between the gender of the IBS patients and depression (Odds ratio = 0.95, $\chi^2 = 0.02$, $P = 0.89$) (Table 2). Even though

Table 1 Relationship between IBS and depression

IBS	Depressed *n* (%)	Not depressed *n* (%)	Total (*n*)
Yes	75 (56.8)	57 (43.2)	132
No	54 (20.1)	214 (79.9)	268
Total	129 (32.3)	271 (67.7)	400

OR = 5.21, 95% CI = 3.22-8.45, χ^2 = 54.29, P < 0.001.

Table 2 Relationship between depression and gender among the IBS patients

Depression	Male *n* (%)	Female *n* (%)	Total (*n*)
Yes	32 (42.7)	43 (57.3)	75
No	25 (43.9)	32 (56.1)	57
Total	57 (43.2)	75 (56.8)	132

OR = 0.95, 95% CI = 0.45-2.03, χ^2 = 0.02, P = 0.89.

Table 3 Relationship between subtype of irritable bowel syndrome and depression

Subtype	Depression *n* (%)	No depression *n* (%)	Total (*n*)
Constipation	36 (61.0)	23 (39.0)	59
Diarrhoea	31 (53.4)	27 (46.6)	58
Alternator	8 (53.3)	7 (46.7)	15
Total	75 (56.8)	57 (43.2)	132

χ^2 = 0.77, df = 2, P = 0.68.

constipation predominant IBS patients were more likely to be depressed, Table 3 shows that no significant relationship exists between the subtype of IBS and depression (χ^2 = 0.77, P = 0.68). No significant difference was observed between the gender of the population and the subtypes of IBS.

DISCUSSION

Seventy-five (56.8%) IBS patients were depressed compared with 54 (20.1%) of the 268 non-IBS patients fulfilled the DSM Ⅳ criteria for depression. There was a highly significant relationship between IBS and depression (χ^2 = 54.29, Odds ratio = 5.21, P < 0.001). This compares well with western studies that reported 60% psychiatric co-morbidity among their IBS patients who have sought healthcare[17,18]. Abnormal psychologic features have been recorded in a large percentage of patients with IBS. In a study by McDonald and others[19] involving 35 patients with IBS and 32 patients with non-IBS disease, 20% of the non-IBS group had diagnosable psychiatric disorders when compared with 53% of the IBS group. This high prevalence of depression among our cohort is most likely due to the fact that it was a study among a patient population. Most western community-based studies and a recent study done by us among stable students showed that the rate of depression was comparable to that among the non-IBS group[7,17]. Indeed, co-morbidities such as psychological symptoms are recognized factors that affect healthcare seeking of IBS patients[20].

Most investigators have found that psychiatric features predate the onset of bowel symptoms or occur concurrently, suggesting that it is not the bowel symptoms of IBS that induce psychiatric diseases[4]. Many theories have implicated the role of depression in IBS[9,14]. In addition, serotonin and catecholeamines (neurotransmitters that play some role in depression), have been shown to be in excess in patients with IBS[21-23]. Emotional stress can trigger bowel hypermotility both in normal subjects and in patients with IBS[24]. These events are associated with development of both organic and functional abdominal illness[25]. Moreover, the existing evidence suggests that treating depression and anxiety can improve the patient's gastrointestinal distress.

There was no significant difference between the gender of the IBS patients and depression, implying that both men and women are equally prone to depression. Even though, a trend was observed in constipation-predominant IBS and depression, there was no statistically significant relationship (P = 0.68). This finding agrees with that by Whitehead *et al*[26].

IBS is significantly associated with major depression, but not with gender and bowel subtypes of the patients. Patients with IBS need to be evaluated for depression due to the highly significant relationship that exists between the two conditions.

The Rome Ⅱ criteria has been widely validated in the western world and found to have a positive predictive value of 98%[27]. In our setting however, where a lot of helminthic and protozoa infestations exist, it is unclear whether the subjects fulfilling these criteria truly have IBS or not. A subset of the patients who had their stools evaluated for these infestations did not change the pre-examination diagnosis of IBS. This is a subject for further evaluations.

ACKNOWLEDGMENTS

We are grateful to the chief executives of the three hospitals, heads of departments of the GOP clinics of the three hospitals for using their patients.

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COMMENTS

Background

Irritable bowel syndrome (IBS) has been widely studied in the western world and many pathophysiologic mechanisms have been provided to explain the constellation of symptoms. Along with the putative pathophysiologic mechanisms of post-infectious inflammatory disorder, disordered intestinal motility and visceral hypersensitivity, there has been much discussion regarding the brain-gut interaction and the influence of behavioral or psychiatric conditions on these symptoms.

Innovations and breakthroughs

IBS is known to co-exist with some psychiatric disorders such as depression, anxiety disorders, somatoform disorders and sexual dysfunction (e.g., dyspareunia). The situation is however, not known in African populations. This study aimed at determining the prevalence of depression among African IBS patients.

Applications

IBS is significantly associated with major depression. Patients with IBS need to be evaluated for depression due to the highly significant relationship between the two conditions. The Rome criteria has been widely validated in the Western world and found to have positive predictive value of 98%. It is necessary to validate this criteria in the African setting, where a lot of helminthic and protozoa infestations exist that may be confused with IBS.

Terminology

IBS is a chronic disorder of unknown etiology clinically consisting of altered bowel habits, abdominal pain and the absence of any detectable organic pathologic process. Depression is a state of low mood, loss of interest and energy that may result in suicidal ideation or act.

Peer review

This is a well-done and interesting study on co-expression of depression and IBS in an African population.

S- Editor Wang GP **L- Editor** Ma JY **E- Editor** Bi L

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World J Gastroenterol 2006 December 28; 12(48): 7848-7851
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Novel *MLH1* frameshift mutation in an extended hereditary nonpolyposis colorectal cancer family

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Received: 2006-05-23 Accepted: 2006-10-14

Abstract

AIM: To present novel frameshift mutation c.31delC [p.L11X] in the *MLH1* gene identified in an extended Bulgarian hereditary non-polyposis colorectal cancer (HNPCC) family and to analyze the molecular and clinical findings within the pedigree concerning the proposal of adequate individual prophylactic strategy for all mutation carriers.

METHODS: The pedigree of the family consists of 42 members in four generations. Search for mutations in the *MLH1* and *hMSH2* genes was performed in the proband. After PCR amplification of all exons including flanking intronic regions, amplicons were directly sequenced.

RESULTS: The mutation was found in nine from the thirteen pedigree members who signed informed consent to participate in the study. In three adenocarcinomas, microsatellite instability and lack of the MLH1 protein expression were detected. The only one tubulovillous adenoma analyzed was microsatellite stable and the MLH1 protein showed an intact staining.

CONCLUSION: The newly described mutation c.31delC is HNPCC causative. Besides the typical clinical features of the syndrome, we found a specific pathologic manifestation such as moderate to high differentiated adenocarcinomas of the colon. One of the mutation carriers developed a benign giant cell soft tissue tumor. The primary tumor localizations were frequently extracolonic and detailed yearly gastrointestinal and gynecological examinations have been proposed to the mutation carriers. We emphasize the importance of including the HNPCC genetic counseling and testing as well in the following surveillance of all patients at risk in the services covered by the health insurance in Bulgaria.

Key words: Colon cancer; Hereditary non-polyposis colorectal cancer; *MLH1*; Microsatellite instability

Kadiyska TK, Kaneva RP, Nedin DG, Alexandrova AB, Gegova AT, Lalchev SG, Christova T, Mitev VI, Horst J, Bogdanova N, Kremensky IM. Novel *MLH1* frameshift mutation in an extended hereditary nonpolyposis colorectal cancer family. *World J Gastroenterol* 2006; 12(48): 7848-7851

http://www.wjgnet.com/1007-9327/12/7848.asp

INTRODUCTION

Hereditary non-polyposis colorectal cancer (HNPCC) or Lynch syndrome is the most common type of hereditary colorectal cancer (CRC), which accounts for about 1% to 3% of all cases with CRC[1] and may be caused by germline mutations in DNA mismatch repair (MMR) genes. Mutations in the *MLH1*[2,3] and *hMSH2*[4,5] genes are responsible for the disease in the majority of HNPCC families. Some mutations have been found to be common in many population studies, whereas others are rare or unique[6] (http://www.insight-group.org/). When a predisposing mutation is found in the proband, the carrier status of the first degree relatives might be clarified after genetic counseling and signing of informed consent[7]. This process is hard and delicate. One of the most frequently observed problems is the anxiety from the result. For all mutation carriers, a prophylactic program is proposed[8]. In Bulgaria, the genetic counseling and testing in hereditary cancer syndromes and the following surveillance for the individuals at risk are not defined or covered by the health insurance. In the period of 1998-2006 the genetic counseling and testing have been financially supported by the National Science Fund

research grants. Our team analyzes individually the pedigrees with heterogenic localizations and particular clinical features, together with the official result from the DNA analysis. Mutation carriers receive information about the exact prophylactic exams to take and the recommended frequency.

In this paper we report a novel frameshift (c.31delC [p.L11X]) mutation in the *MLH1* gene due to a deletion of a cytosine at nucleotide position c.31 in the first exon of the gene in a proband of an extended Bulgarian HNPCC family, fulfilling the Amsterdam criteria. The aim of this study was to analyze the molecular and clinical findings within the pedigree concerning the proposal of adequate individual prophylactic strategy for all mutation carriers.

MATERIALS AND METHODS

The pedigree of the family consists of 42 members in four generations (Figure 1). The proband, a 39 year old man (Ⅲ-7) was operated because of cancer of the cecum. In the family, 15 members were operated on 21 malignant tumors (15 colorectal and 6 extracolonic). The mean age of the first malignant localization was 55 years for the first generation, 63 years for the second generation, 38 years for the third generation and 25 years for the last generation.

A set of five polymorphic markers-BAT26, D2S123, D5S346, D18S35 and FGA, previously found as the most informative in our group of HNPCC patients, have been selected for analysis of microsatellite instability (MSI). Both normal and tumor tissue DNA samples were amplified for the five markers and electrophoresis was performed on an automated fluorescence sequencer (ALF Express, Pharmacia). MSI analysis was possible in four patients (Table 1).

Search for mutations in the *MLH1* and *hMSH2* genes was performed in the proband Ⅲ-7. After PCR amplification of all exons including flanking intronic regions, amplicons were sequenced in both directions using the ABI PRISM Dye terminator cycle sequencing reaction kit (Applied Biosystems Foster City, CA) and ABI-310 Genetic Analyzer. The additional pedigree members were tested only for the presence of the identified mutation in the *MLH1* gene.

In order to investigate the effect of this mutation on the MLH1 protein expression, we chose the immunohistochemistry (IHC) assay. This analysis was performed in four patients with available paraffin-embedded specimens (Table 1). The rabbit polyclonal antibody against the C-terminus of the MLH1 protein (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:100 dilutions was used, following the manufacturer's protocol, with minor modifications. Two investigators assessed the slides for MLH1 staining independently. Informed consent for DNA analysis was obtained from individuals included in the study.

RESULTS

We identified a novel frameshift mutation in the *MLH1* gene, due to a deletion of a cytosine (c.31delC [p.L11X]) leading to a stop codon 16 (TGA), 18 bases downstream (Figure 2). The mutation was found in nine out of the thirteen pedigree members who signed informed consent to participate in the study (Table 1). Individual Ⅱ-1 developed a colorectal cancer at the age of 80 years. Now he is 96 years old and none of his descendents developed cancer. The *MLH1* mutation c.31delC was excluded in this family member.

Table 1 Diagnosis, microsatellite instability, c.31delC mutation and immunohistochemical analysis of ten affected and six healthy at risk relatives in the hereditary non-polyposis colorectal cancer (HNPCC) family

Family member	Diagnosis	Histological data	MSI Analysis	c.31delC mutation	MLH1 expression
Ⅱ-1	Colon carcinoma	NT	NT	No	NT
Ⅱ-3	Colon carcinoma	+	NT	NT	NT
Ⅲ-6	Colon carcinoma	+	NT	NT	NT
Ⅲ-7	Colon carcinoma	+	MSI	Yes	NS
Ⅲ-8	Endometrial carcinoma	+	NT	Yes	NT
Ⅲ-12	Healthy, at risk	NT	NT	Yes	NT
Ⅳ-13	Healthy, at risk	NT	NT	Yes	NT
Ⅳ-14	Colon carcinoma	+	MSI	Yes	NS
Ⅳ-15	Tubulovillous Adenoma	+	MSS	Yes	PS
Ⅳ-16	Healthy, at risk	NT	NT	No	NT
Ⅳ-17	Healthy, at risk	NT	NT	Yes	NT
Ⅳ-18	Colon carcinoma	+	NT	Yes	NT
Ⅳ-19	Healthy, at risk	NT	NT	No	NT
Ⅳ-20	Healthy, at risk	NT	NT	No	NT
Ⅳ-21	Mixed germ cell tumor	+	NT	NT	NT
Ⅳ-22	Colon carcinoma	+	MSI	Yes	NS

NT: not tested; +: available; MSI: microsatellite instability of the tumor; MSS: microsatellite stability of the tumor; Yes: mutation was present; No: mutation was absent; PS: positive staining; NS: negative staining.

All three adenocarcinomas available from CRC patients for MSI analysis showed high instability. The IHC assay showed lack of expression of the MLH1 protein in these tumors. In contrast, microsatellite stability was characteristic of the tubulovillous adenoma and ICH of the tumor showed an intact nuclear staining.

From the remaining 23 members of this branch of the family, fourteen were operated on 20 different malignant tumors, four adenomas and one benign giant cell tumor of soft tissues. Synchronous tumors were found in three individuals, and metachronous in five. Eight colorectal cancers were right sided, three were left sided and no information on the exact tumor localization of the remaining three was available. Only one of the left sided colorectal tumors was the primary localization. The histomorphological study showed that all malignant colorectal tumors were adenocarcinomas, two lowly and nine moderately to highly differentiated. Mucinous production (predominantly extracellular type) was detected in all cases.

DISCUSSION

In the present study we describe a novel *MLH1* mutation c.31delC in relation to the HNPCC phenotype and

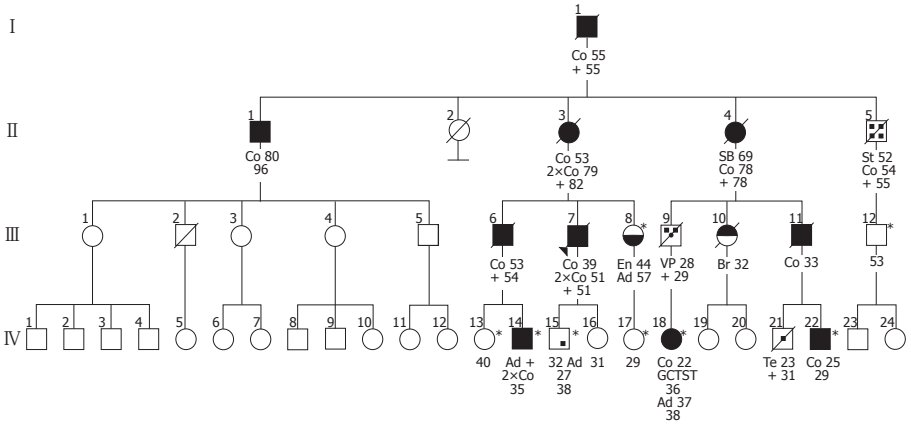

Figure 1 Pedigree tree of the investigated family. Spouses, some of the unaffected and clinically undefined individuals are not presented in the figure. Co: colorectal cancer; VP: vater's Papilla cancer; SB: small bowel cancer; En: endometrial cancer; St: stomach cancer; Br: breast cancer; Te: testicular GCT; Ad: adenoma; GCTST: germ cell tumor of the soft tissue; *healthy mutation carriers.

MLH1 protein expression in colon tumors. Frameshift mutations are frequently found in both *MLH1* and *hMSH2* genes. Bisgaard *et al*[9] identified a mutation in the *MLH1* gene, c.9delC, resulting in a premature stop at the same location (codon 16) as in our study. In the family a skipped generation was present. However the authors conclude that stop codon and exon deletion mutations can be implemented for predictive testing without further analysis. In our study ICH and MSI analyses were used additionally to evaluate the role of this mutation in three adenocarcinomas and one adenoma.

Our genetic analysis showed that the CRC in Ⅱ-1 was not due to the mutation c.31delC. The late age of cancer development (80 years) in this patient, lack of metachronous carcinomas 15 years later and the lack of affected descendents support the sporadic origin of the malignant tumor in this case. Thanks to our study, this branch of the family was relieved of the excessive fear of inherited cancer. Patient Ⅳ-18 developed mixed germ cell tumor (GCT). He provided detailed clinical and genealogical information before his death but refused DNA analysis. The relation between c.31delC/*MLH1* and the risk of breast cancer could not be confirmed, since both daughters of Ⅲ-10 were not carriers of the mutation.

We found the typical features for HNPCC as early age of cancer development, dominant inheritance, high MSI and lack of detectable protein expression in the adenocarcinomas. The colorectal cancers were the most frequent lesions in the family, predominantly right sided, with extracellular mucinous production, presence of synchronous and metachronous tumors. All these observations support the data published by others[10-12]. Interesting findings were the anticipation in the last three generations and the moderate to high colorectal tumor differentiation. High differentiation has been described as specific for Chinese HNPCC families only[13].

The MLH1 protein expression in patient Ⅳ-15 was retained and the tumor showed microsatellite stability (MSS). Protein expression in a mutation carrier with adenoma was described by Stormorken *et al*[14]. The authors speculated that this event might be due to the sporadic origin of the adenoma or that the tumor did not reach the stage of protein loss. The early stage of the tumor diagnosis in our case might explain the presence of intact MLH1 protein, due to the proper functioning of the second, unaffected *MLH1* copy.

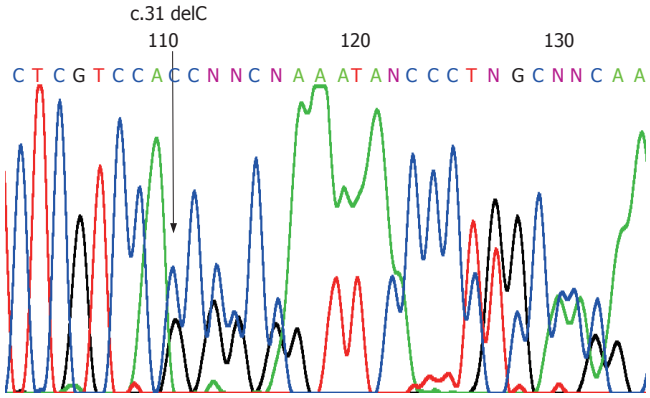

Figure 2 Direct sequencing of the *MLH1* exon 1, demonstrating the mutation c.31delC. The arrow indicates the position of the C deletion.

The observed extracolonic malignant tumor localizations related to this mutation were in endometrium, stomach, Vater's papilla and small bowel. The last two are relatively rarely associated with HNPCC[15] and may be missed by the routine endoscopy.

Besides the tumors involved in the HNPCC spectrum, one of the family members (Ⅳ-23) developed a benign giant cell soft tissue tumor of the third finger, fourteen years after the primary colon localization. We have no data about the finding of such a metachronous tumor in other HNPCC families.

We conclude that the newly described mutation c.31delC is HNPCC causative. Besides the typical clinical features of the syndrome, we have found a specific pathologic manifestation as moderately to highly differentiated adenocarcinomas of the colon. The primary tumor localizations are frequently extracolonic and detailed yearly gastrointestinal and gynecological examinations have been proposed to the mutation carriers. We emphasize the importance of including the HNPCC genetic counseling and testing as well in the following surveillance of all patients at risk in the services covered by the health insurance in Bulgaria.

ACKNOWLEDGMENTS

We thank all family members who agreed to attend our

study. We also thank Dr. Traykova who kindly provided us with paraffin-embedded tissues and medical records.

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S- Editor Wang GP **L- Editor** Zhu LH **E- Editor** Bi L

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World J Gastroenterol 2006 December 28; 12(48): 7852-7858
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Expression patterns and action analysis of genes associated with physiological responses during rat liver regeneration: Innate immune response

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Received: 2006-08-25 Accepted: 2006-10-09

Abstract

AIM: To study the relationship between innate immune response and liver regeneration (LR) at transcriptional level.

METHODS: Genes associated with innate immunity response were obtained by collecting the data from databases and retrieving articles. Gene expression changes in rat regenerating liver were detected by rat genome 230 2.0 array.

RESULTS: A total of 85 genes were found to be associated with LR. The initially and totally expressed number of genes at the phases of initiation [0.5-4 h after partial hepatectomy (PH)], transition from G0 to G1 (4-6 h after PH), cell proliferation (6-66 h after PH), cell differentiation and structure-function reconstruction (66-168 h after PH) was 36, 9, 47, 4 and 36, 26, 78, 50, respectively, illustrating that the associated genes were mainly triggered at the initial phase of LR and worked at different phases. According to their expression similarity, these genes were classified into 5 types: 41 up-regulated, 4 predominantly up-regulated, 26 down-regulated, 6 predominantly down-regulated, and 8 approximately up/down-regulated genes, respectively. The expression of these genes was up-regulated 350 times and down-regulated 129 times respectively, demonstrating that the expression of most genes was enhanced while the expression of a small number of genes was decreased during LR. Their time relevance was classified into 14 groups, showing that the cellular physiological and biochemical activities during LR were staggered. According to the gene expression patterns, they were classified into 28 types, indicating that the cellular physiological and biochemical activities were diverse and complicated during LR.

CONCLUSION: Congenital cellular immunity is enhanced mainly in the forepart, prophase and anaphase of LR while congenital molecular immunity is increased dominantly in the forepart and anaphase of LR. A total of 85 genes associated with LR play an important role in innate immunity.

Key words: Partial hepatectomy; Rat genome 230 2.0 array; Innate immune response; Genes associated with liver regeneration

Chen GW, Zhang MZ, Zhao LF, Xu CS. Expression patterns and action analysis of genes associated with physiological responses during rat liver regeneration: Innate immune response. *World J Gastroenterol* 2006; 12(48): 7852-7858

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INTRODUCTION

Organisms can resist and remove endogenous and exogenous poisons *via* their innate immune cells and other factors. This process is known as innate immune response[1], a self-protection mechanism of living organisms which is absolutely indispensable to their survival[2]. Innate immune responses consist of three parts, namely barrier of self-tissue, innate cellular immunity and innate molecular immunity. Tissue barrier can excrete antibacterial and bactericidal matters to kill pathogens, innate cellular immunity can not only remove pathogens invading body *via* immune cells but also clear the broken, dead and abnormal cells, while innate molecular immunity can demolish and dissolve injurious substances *via* active molecules and cytokines[3]. Liver containing NK cells, T lymphocytes, macrophages, *etc*, is an important organ where innate immune response takes place[4]. After partial hepatectomy (PH), liver undergoes severe injury. How the remnant liver cells are protected by the innate immune system deserves intensive study[5].

In addition, PH[6] can activate the remaining hepatocytes to rapidly proliferate and compensate for the

loss of liver mass, which is known as liver regeneration (LR)[7,8]. Based on the cellular physiological activities, the regeneration proceeding is usually categorized into four stages: initiation (0.5-4 h after PH), transition from G0 to G1 (4-6 h after PH), cell proliferation (6-66 h after PH), cell differentiation and reorganization of the structure-function (66-168 h after PH)[8]. According to the time course, it can be classified into four phases: forepart (0.5-4 h after PH), prophase (6-12 h after PH), metaphase (16-66 h after PH), and anaphase (72-168 h after PH)[9], involving many physiological and biochemical events, such as cell activation, cell de-differentiation, cell proliferation and its regulation, cell re-differentiation, reorganization of structure-function[10], which are regulated by many factors including innate immune response. The action of genes associated with liver diseases caused by hepatitis virus infection and pathogen infection during LR, has been analyzed[11-13]. In the present study, rat genome 230 2.0 array containing 151 genes involved in innate immune response was used to detect the gene expression changes in regenerating liver after 2/3 hepatectomy as previously described[14-17] in order to investigate the relevance between LR and innate immune response at transcriptional level. The expression changes, patterns and action of these genes during LR were primarily analyzed. Our results indicate that 85 out of the 151 genes are associated with LR[18].

MATERIALS AND METHODS

Regenerating liver preparation

Healthy SD rats weighing 200-250 g were obtained from the Animal Center of Henan Normal University. The rats were divided into groups at random, 6 rats in each group (male: female = 1:1). PH was performed as previously described[6], the left and middle lobes of liver were removed. The rats were killed by cervical vertebra dislocation at 0.5, 1, 2, 4, 6, 8, 12, 16, 18, 24, 30, 36, 42, 48, 54, 60, 66, 72, 96, 120, 144 and 168 h after PH and the regenerating livers were observed at corresponding time points. The livers were rinsed three times in PBS at 4℃, then 100-200 mg liver tissue was taken from middle part of the right lobe. Six samples were collected from each group and mixed into 1-2 g (0.1-0.2 g × 6) liver tissue, then stored at -80℃. The sham-operation (SO) groups underwent the same PH without removal of the liver lobes. The animal protection laws of China were strictly followed.

RNA isolation and purification

Total RNA was isolated from frozen livers according to the manual of Trizol kit (Invittrogen)[19] and then purified based on the guide of RNeasy mini kit (Qiagen)[20]. Agarose electrophoresis (180V, 0.5h) showed that total RNA sample exhibited a 2:1 ratio of 28S to 18S rRNA intensities. Total RNA concentration and purity were estimated by optical density measurements at 260/280 nm[21].

cDNA, cRNA synthesis and purification

Total RNA (1-8 μg) was used as a template for cDNA synthesis. cDNA and cRNA synthesis was proceeded as previously described[16]. cRNA labeled with biotin was synthesized using 12 μL synthesized cDNA as a template, cDNA and cRNA were purified[16]. Measurement of concentration, purity and quality of cDNA and cRNA was performed as previously reported[21].

cRNA fragmentation and microarray detection

Fifteen μL (1 μg/μL) cRNA incubated with 5 × fragmentation buffer at 94℃ for 35 min was digested into 35-200 bp fragments. The hybridization buffer was added to the prehybridized Rat Genome 230 2.0 microarry produced by Affymetrix, and then hybridization was carried out for 16 h at 45℃ on a rotary mixer at 60 rpm. The microarray was washed and stained by GeneChip fluidics station 450 (Affymetrix Inc., USA). The chips were scanned by GeneChip scan 3000 (Affymetrix Inc., USA), and the signal values of gene expression were observed[17].

Microarray data analysis

The normalized signal values, signal detections (P, A, M) and experiment/control (Ri) were obtained by quantifying and normalizing the signal values using GCOS1.2[17].

Normalisation of microarray data

To minimize the errors in microarray analysis, each analysis was performed three times by rat genome 230 2.0 microarray. Results with a maximal total ratio (R^m) and an average of three housekeeping genes β-actin, hexokinase and glyseraldehyde-3-phosphate dehydrogenase approaching 1.0 (R^h) were taken as a reference. Modified data were generated by applying a correction factor (R^m/R^h) multiplying the ratio of every gene in Rh at each time point. To remove spurious gene expression changes resulting from errors in the microarray analysis, the gene expression profiles at 0-4 h, 6-12 h and 12-24 h after PH were reorganized by normalization analysis program (NAP) software according to the cell cycle progression of regenerating hepatocytes. Data statistics and cluster analysis were done using the GeneMath, GeneSpring, Microsoft Excel softwares[17,22,23].

Identification of genes associated with liver regeneration

First, the nomenclature of innate immune response was adopted from the GENEONTOLOGY database (www.geneontology.org) and input into NCBI (www.ncbi.nlm.nih.gov) and RGD (rgd.mcw.edu) to identify the rat, mouse and human genes associated with the biological process. According to the maps of biological pathways embodied by GENMAPP (www.genmapp.org), KEGG (www.genome.jp /kegg/pathway.html#amino) and BIOCARTA (www.biocarta.com/genes/index.asp), genes associated with innate immune response were collated. The results of this analysis were codified and compared with those obtained for humans and mice in order to identify human and mouse genes which are different from those of rats. Comparing these genes with the analysis output of rat genome 230 2.0 array, genes showing more than twofold change in expression level as meaningful expression changes[18], were referred to as rat homologous or rat specific genes associated with innate immune response

Table 1 Expression of 85 innate immune response-associated genes during rat liver regeneration

Gene Abbr.	Associated to others	Fold difference	Gene Abbr.	Associated to others	Fold difference	Gene Abbr.	Associated to others	Fold difference	Gene Abbr.	Associated to others	Fold difference
Innate immune cells			Colec12		3.9	Bdkrb2	3	0.4	Cxcl10		0.3, 9.2
1 Macrophage			Crp		0.5	[1]Il1b	1	0.4	Cxcl12		0.2
Adora2a		0.5	Hrh1	1	0.5, 9.9	Il1f5	1	0.4, 2.8	Darc		0.4, 8.5
Anxa1		4.3	Hrh4	1	7.5	Il1r1	1	0.5	Others		
Cebpb		3.1	Mcpt6	1, 2	0.2	Il1rn	1	16.3	Parp4		0.5
Clec7a		0.2	Nr3c1		4.7	Il2		0.3, 3.5	Alox5		0.2, 2.5
Cybb		2.5	Spp1		0.5, 2.7	Il5		3.5	Alox5ap		4.9
Ereg	2	0.4	Nfatc4		0.5	Sarm1		0.2, 4.3	Apoe		0.1
Ltb4r		0.5, 8.7	Innate immune effectors			Sele	3	12.9	Casp12		0.4,2.6
Mif		3.2	4 Complement system			b Interferon and related factors			Dmbt1		9.8
Pap		68.6	C1qa		0.3	Ifnk		0.1, 5.7	Hck		0.4
Pla2g4a		2	C1qr1	1, 2	5.5	Ddx58		11.8	Map2k3		0.4
Ptgs2		0.1, 2.1	C2		2.1	Irf3		2.6	Prkca		4.6
S100a8		6.5	[1]C3		0.2	Mx2		9.4	Ptafr		7.1
S100a9		4.9	C3ar1		0.3, 2.3	c Iumor necrosis factor and related factors			Reg3a		0.1, 64.1
Tgfb1	2, 3	4.0	C4a		0.5	Ager		0.4	Reg3g		0.3, 7.5
2 NK cell			C4bpa		2.0	Myd88		2.1			
Ncr3		0.3	C5ar1		0.4, 2.6	Tnf	1	3.2			
Ptger3		0.2	Cfh		2.5	d Chemotactic numerator and telated factors					
Rela		0.5	Cfi		6.4	Ccl17		0.1			
Ripk2		0.4	Cr2	3	6.0	Ccl19		3.9			
Tlr2		10.6	Crry		2.4	Ccl2		128.0			
Tlr4		0.5	Masp1		3.0	Ccl20		8.0			
3 Other cells			Mbl2		0.2	Ccl24		4.0			
Aoc3	1	6.1	5 Cell factors			[1]Ccl4		0.2, 3.0			
Atrn	1	4.4	a Interlenkin and related factors			Ccl7		22.6			
Clu	1, 2	3.0	Bcl3	1	0.4	Ccr1		0.4,.24.9			

[1]Reported genes associated with liver regeneration; associated to others: genes are involved in another kind of the responses besides one kind of innate immune responses; others: other genes associated with innate immune response, but cannot be clearly categorized.

under evaluation. Genes displaying reproducible results in three independent analyses with the chip and more than twofold change in expression level at least at one time point during LR with a significant difference ($P \leqslant 0.01 < 0.05$) or an extremely significant difference ($P \leqslant 0.01$) between PH and SO, were referred to as genes associated with liver regeneration.

RESULTS

Expression changes of innate immune response-associated genes during liver regeneration

According to the data from databases at NCBI, GENEMAP, KEGG and BIOCARTA, a total of 275 genes are involved in innate immune response, of which 151 are contained in the rat genome 230 2.0 array. In the resent study, 85 out of these 151 genes revealed meaningful expression changes at least at one time point after PH, showing a significant difference or an extremely significant difference in expression when PH was compared with SO and reproducible results checked by three detects with rat genome 230 2.0 array, suggesting that the genes were associated with LR. Their expression was up-regulated 2 to 128 times and down-regulated 2 to 10 times of the control (Table 1). The expression was up-regulated in 41, down-regulated in 26, and up/down-regulated in 18 genes, respectively, during LR. The expression of these 85 genes was up-regulated 350 times and down-regulated 129 times (Figure 1A). At the initiation stage of LR (0.5-4 h after PH), the expression was up-regulated in 28 and down-regulated in 8 genes. At the transition phase from G0 to G1 (4-6 h after PH), 21 was up-regulated in 21 and down-regulated in 5 genes. At cell proliferation period (6-66 h after PH), the expression was up-regulated in 42, down-regulated in 29, and up/down-regulated in 8 genes, respectively. At cell differentiation and reorganization of the structure-function stage (66-168 h after PH), the expression was up-regulated in 32, down-regulated in 14, and up/down-regulated in 4 genes, respectively (Figure 1B).

Initiation expression time of innate immune response-associated genes during liver regeneration

At each time point of LR, the number of initially and totally up-regulated, down-regulated genes respectively was both 10 and 2 at 0.5 h; 7,6 and 15, 6 at 1 h; 7, 0 and 18, 1 at 2 h; 4, 0 and 20, 1 at 4 h; 1, 4 and 14, 5 at 6 h; 0, 0 and 16, 3 at 8 h ; 1, 0 and 19, 3 at 12 h ; 7, 4 and 19, 4 at 16 h ; 3, 9 and 19, 14 at 18 h ; 1, 0 and 17, 14 at 24 h ; 0, 3 and 12, 8 at 30 h ; 1, 3 and 19, 9 at 36 h ; 1, 1 and 15, 5 at 42 h ; 2, 1 and 26, 12 at 48 h; 0, 2 and 15, 11 at 54 h ; 0, 1 and 16, 7 at 60 h ; 0, 0 and 12, 4 at 66 h ; 0, 0 and 10, 4 at 72 h ; 1, 0 and 14, 7 at 96 h ; 3, 0 and 18, 6 at 120 h ; 0, 0 and 12, 5 at 144 h ; 0, 0 and 14, 6 at 166 h (Figure 2). Generally, gene expression changes occurred during the

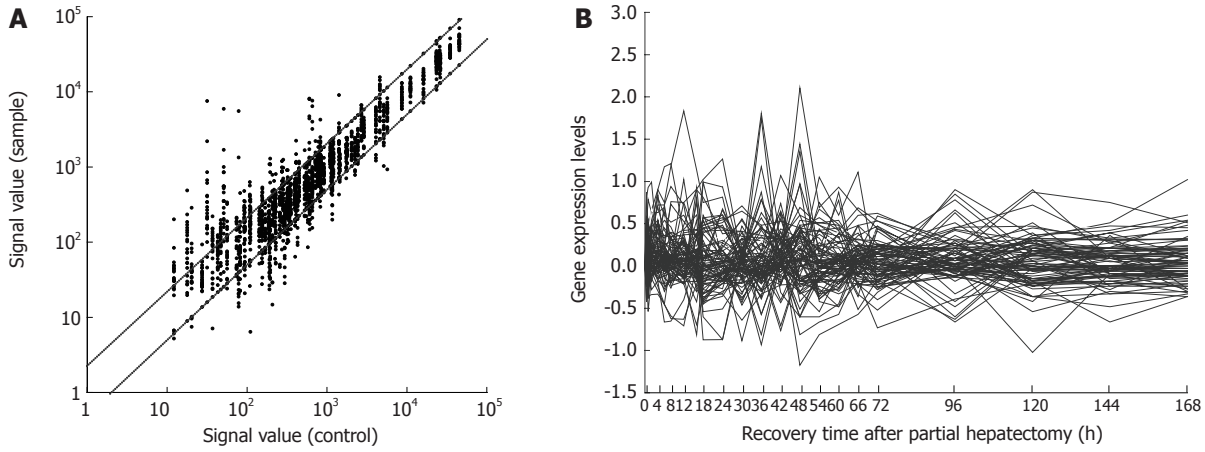

Figure 1 Expression frequency (**A**) and changes (**B**) of 85 innate immune response-associated genes during rat liver regeneration. Data detected by rat genome 230 2.0 array were analyzed and graphed by Microsoft Excel. The dots above bias indicate that the expression of genes was increased more than two folds and up-regulated 350 times, the dots under bias indicate that the expression of genes was decreased more than two folds and down-regulated 129 times, the dots between biases indicate that the expression of genes has no alteration. The expression of 59 genes was increased 2-128 folds, while the expression of 44 genes was increased 2-10 folds.

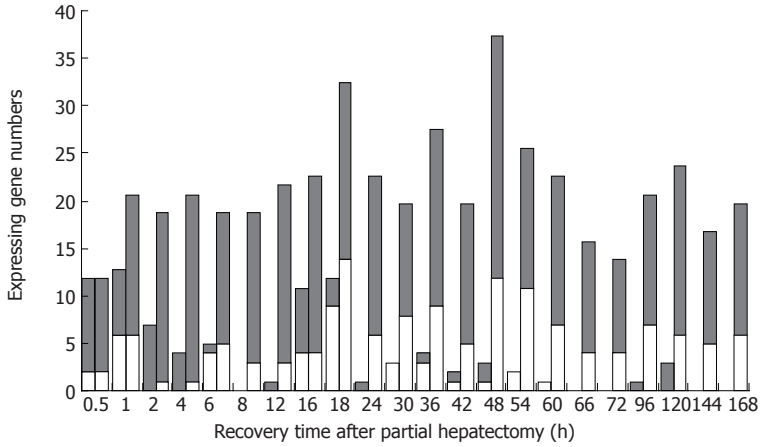

Figure 2 Initial and total expression profiles of 85 innate immune response-associated genes at each time point of liver regeneration. Grey bars: up-regulated expression gene; white bars: down-regulated expression gene; blank bars: initially expressed genes in which up-regulated genes are predominant in the forepart, and down-regulated genes in the prophase and metaphase, whereas very few in the anaphase; dotted bars: the total number of expressed genes, in which the expression of some genes is up-regulated and the expression of others is down-regulated during LR.

whole LR. The expression of these genes was up-regulated 350 times and down-regulated 129 times. The expression of the genes was predominantly initially up-regulated in the forepart, and initially down-regulated in the prophase and metaphase, whereas the initial expression of very few genes was observed in the anaphase.

Expression similarity and time relevance of innate immune response-associated genes during liver regeneration

Based on their similar expression, the 85 genes during LR could be divided into 41 up-regulated, 4 predominantly up-regulated, 26 down-regulated, 6 predominantly down-regulated, and 8 up/down-regulated genes, respectively (Figure 3). Based on their time relevance, they could also be classified into 14 groups (0.5 h, 1 and 66 h, 2 h, 4 and 8 h, 12 and 36 h, 16 and 96 h, 18 and 24 h, 30 and 42 h, 48 h, 54 and 60 h, 72 h, 120 h, 144 and 168 h), in which their expression was up- and down-regulated at 10 and 2 h, 27 and 10 h, 18 and 1 h, 20 and 1 h, 30 and 8 h, 38 and 12 h, 33 and 11 h, 36 and 20 h, 26 and 12 h, 31 and 18 h, 10 and 4 h, 18 and 6 h, 26 and 11 h (Figure 3). The up-regulated expression of genes was mainly associated with cellular immunity. The down-regulated expression of genes was significantly associated with molecular immunity.

Expression patterns of innate immune response-associated genes during liver regeneration

According to their expression changes during LR, the patterns of the above 85 genes might be categorized into 28 types: 5 up-regulated genes at one time point, i.e. at 4, 48, 96, 120 h after PH (Figure 4A); 4 up-regulated genes at two time points, i.e. at 12 and 60 h, 42 and 120 h, 16 and 42 h (Figure 4B); 1 up-regulated gene at three time points (Figure 4B); 5 up-regulated genes at more time points (Figure 4B); 3 up-regulated genes at one phase, i.e. at 0.5-8 h, 4-8 h, 120-168 h (Figure 4C); 1 up-regulated gene at two phases, i.e. at 16-36 h, 42-48 h (Figure 4C); 1 up-regulated gene at three phases (Figure 4C); 1 up-regulated gene at more phases (Figure 4C); 1 up-regulated gene at one time point/one phase, i.e. at 120 and 2-72 h, 48 and 2-24 h, 18 and 48-60 h, 42 and 120-168 h (Figure 4D); 1 up-regulated gene at two time points/three phases (Figure 4E); 1 up-regulated gene at two time points/one phase (Figure 4E); 1 up-regulated at one time point/ three phases (Figure 4E); 1 up-regulated gene at two time points/three phases (Figure 4E); 3 up-regulated genes at two time points/two phases (Figure 4E); 1 up-regulated gene at three time points/ one phase (Figure 4F); 1 up-regulated gene at three time points/two phases (Figure 4F); 3 up-regulated genes at

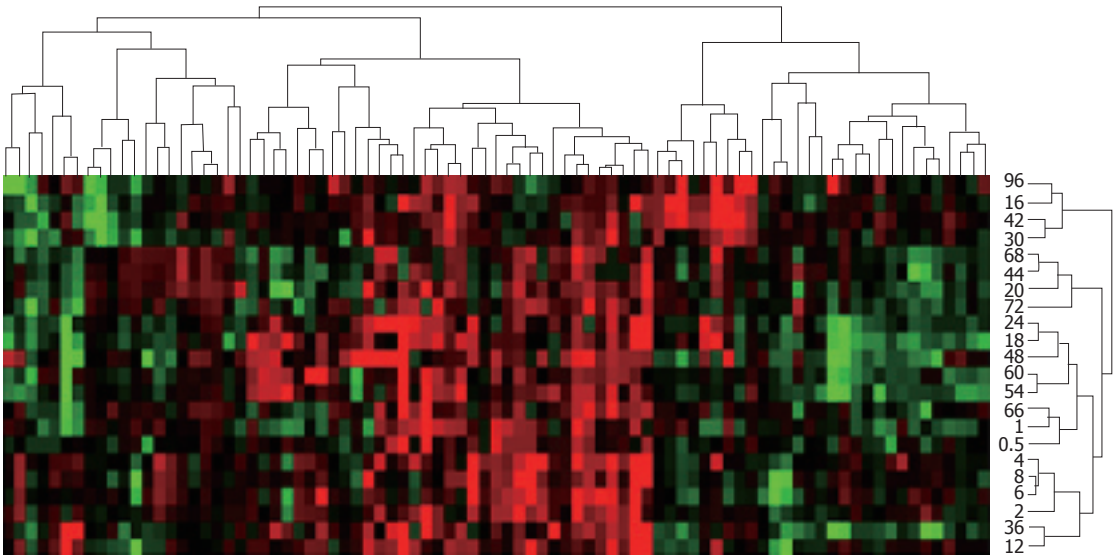

Figure 3 Expression similarity and time relevance clusters of 85 innate immune response-associated genes during liver regeneration. Data detected by rat genome 230 2.0 array were analyzed by H-clustering. Red indicates up-regulated gene expression chiefly associated with cellular immunity; green indicates down-regulated gene expression mainly associated with molecular immunity; black indicates meaningless change in gene expression. The upper and right trees show the expression similarity and time series clusters, by which the above genes were classified into 5 and 14 groups respectively.

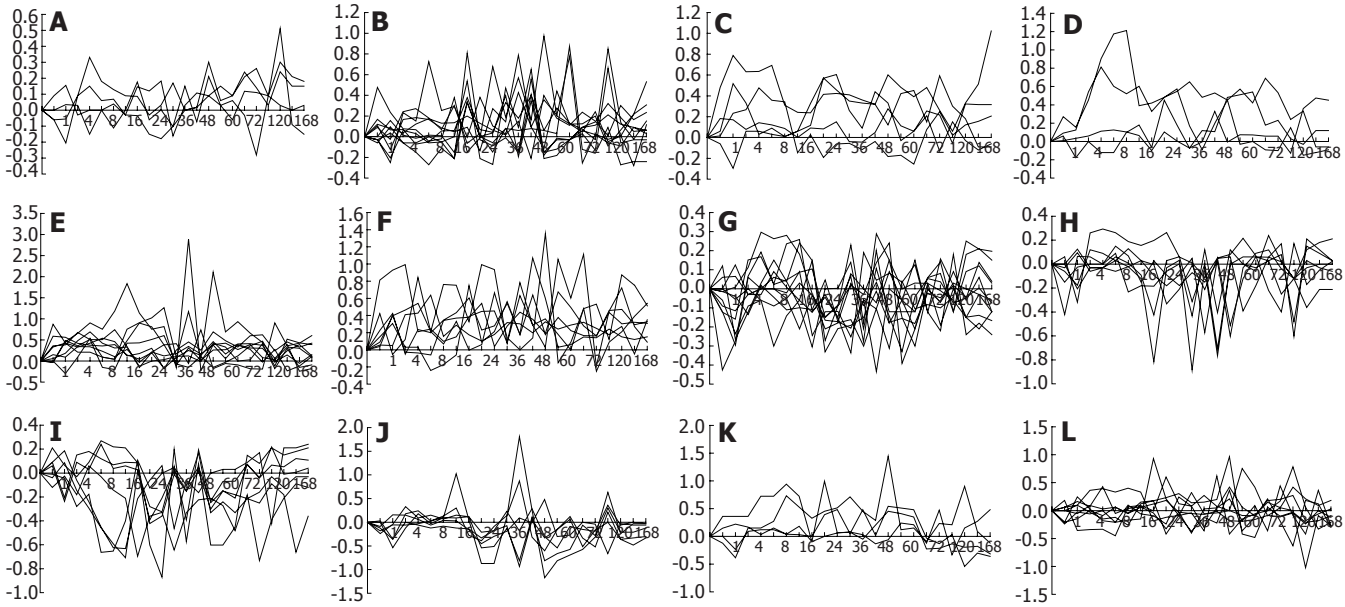

Figure 4 Expression patterns of 85 innate immune response-associated genes during liver regeneration. Twenty-eight expression patterns were obtained by the analysis of data detected by rat genome 230 2.0 array with Microsoft Excel. **A**-**F**: 41 up-regulated genes; **G**-**I**: 26 down-regulated genes; **J**-**L**: 18 up/down-regulated genes. X-axis represents recovery time after PH (h); Y-axis shows logarithm ratio of the signal values of genes at each time point to control.

more phases (Figure 4F); 11 down-regulated genes at one time point, i.e. 0.5, 6, 16, 18, 30, 36, 42, 48, 54, 60 h (Figure 4G); 5 down-regulated genes at two time points, i.e. at 0.5 and 48, 1 and 72, 18 and 54, 30 and 42h (Figure 4H); 1 down-regulated gene at three time points (Figure 4H); 2 down-regulated genes at more time points (Figure 4H); 2 down-regulated genes at two time points/one phase (Figure 4I); 1 down-regulated gene at one phase, i.e. 6-12h (Figure 4I); 2 down-regulated genes at one time point/two phases (Figure 4I); 2 down-regulated genes at two time points/one phase (Figure 4I); 6 first down- and then up-regulated genes (Figure 4J); 4 first up- and then down-regulated genes (Figure 4K); 8 up/down-regulated genes (Figure 4L).

DISCUSSION

Innate immune response, which is a self-protection mechanism formed during the long-term evolutionary process, includes tissue barrier, innate cellular immunity and innate molecular immunity, being closely linked to existence of high animal[2]. Of the proteins associated with innate cellular immunity, seven proteins including toll-like receptor 2 (TLR2) have a role in recognition of pathogens, interferon excretion of NK cells and activation of congenital immune system[24,25]; four proteins including attractin (ATRN) positively regulate antigen representation[26,27]; fifteen proteins including CCAAT/

enhancer binding protein beta (CEBPB) and S100 calcium binding protein A8 (S100A8) promote macrophage phagocytosis[28-32]; macrophage migration inhibitory factor (MIF), also called glycosylation-inhibiting factor, inhibits the function of macrophages[33]; glucocorticoid receptor (NR3C1) inhibits action of dendritic cells[34]. In the present study, the expression of the above genes was identical or similar at some time points, while different at other time points, indicating that they co-regulate cellular immune response. Among them, *cebpb* expression was up-regulated at 0.5-8 h after PH and reached its peak at 1 h, which was 3.1 folds of the control. *s100a8* expression was up-regulated at 2-72 h and 120 h after PH, and reached its peak at 4 h, which was 6.5 folds of the control. *nr3c1* and *tlr2* expression was up-regulated at multiple phases during LR and reached their peak at 168 h and 42 h respectively, which was 4.7 folds and 10.6 folds of the control. *atrn* expression was up-regulated mainly at the metaphase, showing that the highest expression at 42 h was 4.4 folds that of the control. These findings suggest that the genes are the key to innate cellular immunity in regenerating liver.

Of the proteins associated with innate molecular immunity, eleven proteins, such as chemokine C-C motif ligand 2, 4 (CCL2, CCL4), are associated with recognition of pathogens and immune enhancement[35-38]. Two proteins, such as annan-binding lectin serine peptidase 1 (MASP1), activate the complement system[39]. Five proteins including complement component 2 (C2) enhance inflammation[40,41]. Four proteins including interferon regulatory factor 3 (IRF3) interfere with the multiplication of virus[42]. Two proteins including complement component 1 q subcomponent receptor 1 (C1QR1) are responsible for removal of apoptotic cells[43,44]. Ten proteins including interleukin 1 family member 5 delta (IL1F5) have a role in elimination of the pathogens in inflammatory response[45-47]. Three proteins including tumor necrosis factor (TNF) have the function of sterilization by promoting NK cell proliferation[48]. In the present study, the expression changes of the genes encoding these proteins were identical or similar at some time points and different at other time points, suggesting that they can co-modulate innate molecular immunity. *ccl4* expression was up-regulated only at 48 h after PH, which was 3-fold of the control. However, Masson *et al*[38] reported that it is down-regulated at 3 and 12 h. *ccl2* expression was up-regulated at 0.5-1, 12-24, 36, 48-72 and 120 h, and reached its peak at 48 h, which was 128-folds of the control. *c1qr1* expression was up-regulated at multiple time points post PH and reached its peak at 8 h, which was 5.6 folds of the control. *masp1* expression was up-regulated at 42 and 120-168 h and reached its peak at 144 h, which was 3 folds that of the control. These findings indicate that the genes are important in molecular immunity during LR.

In conclusion, the expression changes of congenital immune response-associated genes after rat PH can be analyzed with high-throughput gene expression assay. The congenital immunity is enhanced during LR. Rat Genome 230 2.0 array was a useful tool analyzing the above response at transcriptional level. Nevertheless, DNA→ mRNA→ protein is influenced by various factors including protein interaction. Therefore, such techniques as Northern blotting, protein chip, RNA interference, protein-interaction *etc*, are needed to further test the above results.

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S- Editor Wang GP **L- Editor** Wang XL **E- Editor** Bi L

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World J Gastroenterol 2006 December 28; 12(48): 7859-7863
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Overexpression of Ets-like protein 1 in human esophageal squamous cell carcinoma

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Received: 2006-10-14 Accepted: 2006-11-27

Abstract

AIM: To study the expression pattern of Ets-like protein 1 (Elk-1) in human esophageal squamous cell carcinoma (ESCC) and to analyze its relationship with clinicopathologic parameters.

METHODS: The expression of Elk-1 in fresh esophageal cancer tissues and their corresponding normal mucosae was detected immunohistochemically (IHC) by means of tissue microarray (TMA). Its correlation with clinical characteristics was evaluated and analyzed by univariate analysis. All statistical analyses were performed by SPSS version 13.0.

RESULTS: Expression level of transcription factor Elk-1 increased in 78.5% (84/107) ESCC tissues compared with their matched normal esophageal epithelium. However, the expression of Elk-1 did not show any obvious correlation with degree of differentiation of esophageal carcinoma (in well-differentiated, moderately-differentiated and poorly-differentiated tumors, the increased expression was 7/8, 60/74, and 19/25, respectively, $P > 0.05$). Moreover, no obvious correlation was found with lymph node metastasis and depth of invasion.

CONCLUSION: Increased expression of transcription factor Elk-1 may play an important role in esophageal carcinogenesis.

Key words: Ets-like protein 1; Esophageal squamous cell carcinoma; Immunohistochemistry; Tissue microarray

Chen AG, Yu ZC, Yu XF, Cao WF, Ding F, Liu ZH. Overexpression of Ets-like protein 1 in human esophageal squamous cell carcinoma. *World J Gastroenterol* 2006; 12(48): 7859-7863

http://www.wjgnet.com/1007-9327/12/7859.asp

INTRODUCTION

Esophageal cancer ranks among the 10 most frequent cancers in the world, with a predominant distribution in developing countries. It is one of the most common malignant tumors in China[1,2]. Our previous study showed that genetic susceptibility to esophageal cancer is one of the important causes for the high prevalence and familial aggregation of this disease in some areas of northern China[3]. Ets-like protein 1 (Elk-1) is a member of the ternary complex factor (TCF) subfamily of E twenty-six (ETS)-domain transcription factors[4,5]. The three ternary complex factors (TCFs) Elk-1, Net and Sap-1 form a subfamily of the ETS domain transcription factors. Their characteristic property is the ability to form a ternary nucleoprotein complex with the serum response factor (SRF) over the serum response element (SRE) of the c-*fos* promoter. The molecular mechanisms underlying the function and regulation of these factors have been extensively studied and the TCFs are a paradigm for the study of transcriptional regulation in response to extracellular signalling through the mitogen-activated protein (MAP) kinase pathway. As final effectors of multiple signalling pathways and components of protein complexes on immediate early promoters, they represent key elements in the complex and dynamic regulation of gene expression[6].

Tissue microarray (TMA) was first introduced in 1998[7]. It is a high throughput technique that can significantly accelerate the processing of a large number of tissue specimens with excellent quality, good reliability and the preservation of original tissue. TMA studies can demonstrate their accuracy and reliability compared to those of standard histological techniques and correlate with clinicopathologic information to determine disease progression and prediction of the clinical outcome[8]. It allows simultaneous analysis of many tumors using small diameter cores sampled from larger blocks of tissue, but may be limited by tumor heterogeneity[9]. In this study, we used TMA to investigate the transcription factor Elk-1 in esophageal squamous cell carcinoma (ESCC), including

method for assessing immunohistochemical scoring of microarrays. TMA blocks were constructed from 107 cases of ESCC with corresponding normal tissues, taking two cores from different areas of each tumor and two cores from adjacent esophageal epithelia. Immunohistochemical labelling was performed for Elk-1. The extent and intensity of scoring were determined for each core and the degree of agreement was determined for results from the assessment of two, three or four cores for each case. The results show that TMA is a reliable tool to demonstrate cellular and molecular alterations in ESCC.

In this study, we investigated the protein expression of transcription factor Elk-1 in ESCC. The expression levels of Elk-1 increased in ESCC tissues compared with their normal counterparts. Therefore, Elk-1 might be related to human ESCC and further study on Elk-1 may provide insight into the mechanisms of carcinogenesis of esophagus.

MATERIALS AND METHODS

Materials

Specimens of cancer tissues and matched adjacent normal mucosa were taken from 107 consecutive patients with squamous cell carcinoma of the thoracic esophagus who underwent esophagectomy with regional lymph nodes dissected from July 2005 to April 2006 at the Department of Thoracic Surgery, the First Affiliated Hospital of Anhui Medical University. None of the patients received radiotherapy or chemotherapy before surgery. The patients included 79 men and 28 women with a median age of 60 (range 40-79) years. Fourteen tumors were located in the upper thorax, 60 in the middle thorax and 33 in the lower thorax (Table 1). The removed specimens were stained with hematoxylin and eosin, examined histologically, and then the clinicopathologic stage was determined according to TNM classification.

Table 1 Clinical and histopathological characteristics of patients

Characteristic	*n* (%)
Sex	
Male	79 (73.8)
Female	28 (26.2)
Location of tumor	
Upper thoracic	14 (13.1)
Middle thoracic	60 (56.1)
Lower thoracic	33 (30.8)
Degree of differentiation	
Well-differentiated	8 (7.5)
Moderately-differentiated	74 (69.2)
Poorly-differentiated	25 (23.3)
Depth of invasion	
T1	6 (5.6)
T2	39 (36.4)
T3	62 (58.0)
T4	0 (0)
Lymph node metastasis	
Positive	35 (32.7)
Negative	72 (67.2)

Construction of tissue microarray

The collected samples were fixed with formalin and embedded with paraffin, and then tissue microarray was performed. Two pathologists selected representative areas from each donor tumor block, and then punched cores 1.0 mm in diameter, from the donor blocks, and then transferred these tissue cores to a recipient block using a tissue microarrayer (Beecher Instrument, Silver Spring, Maryland, USA). The resultant tissue microarray was cut into sections and transferred to glass slides for processing of Elk-1 by immunohistochemistry.

Immunohistochemical staining

Immunohistochemical analysis was done retrospectively. Resected esophageal specimens, including both tumor and normal mucosae, were fixed in a 40 g/L formaldehyde solution and embedded in paraffin. Rabbit polyclonal IgG Elk-1 antibody (Santa Cruz Biotech Co, USA) was used in this study (diluted 1:100). Formalin-fixed and paraffin embedded tissue blocks of esophageal tumors were cut into 4-μm thick sections. TMAs were deparaffinized in xylene, rehydrated in ethanol and treated with 30 mL/L H_2O_2 for 30 min to block the endogenous peroxidase activity. Antigen retrieval was achieved by microwaving in 0.01 mol/L citrate buffer (pH 6.0) at 96℃ for 15 min. After incubation with 10% normal goat serum to block non-specific binding, they were then incubated with anti-Elk-1 antibody at 1:100 dilution overnight at 4℃. After antibody was washed in PBS, TMAs were incubated with the secondary antibody and the third antibody (Streptavidin/HRP) according to the manufacturer's instructions. Finally DAB was used as a chromogen and hematoxylin as a counterstain. Negative control was designed by using PBS instead of primary antibody.

Assessment of staining

The percentage of Elk-1-positive tumor cells was determined semiquantitatively by assessing the entire tumor section and scored as: a = 0, < 5% of epithelial cells in the respective lesions; b = 1, 5%-25% of epithelial cells in the respective lesions; c = 2, 26%-50% of epithelial cells in the respective lesions; d = 3, 51%-75% of epithelial cells in the respective lesions; e = 4, > 75% of epithelial cells in the respective lesions. The intensity was graded as: a = 0, negative; b = 1 +, weak; c = 2 +, moderate; d = 3 +, strong. A final score between 0 and 12 was achieved by multiplication of the extent of positivity and intensity[10,11]. Positive staining of more than 5% in cell cytoplasm was defined as positive staining, less than 50% in cell cytoplasm as preserved expression, more than 50% in cell cytoplasm as increased expression[12].

Statistical analysis

Paired-samples T test, chi square test or Fisher's exact probability test was used to assess the association between immunohistochemical features and clinicopathologic characteristics. A P value less than 0.05 was considered statistically significant. All the statistical analyses were performed using the SPSS 13.0 V for Windows.

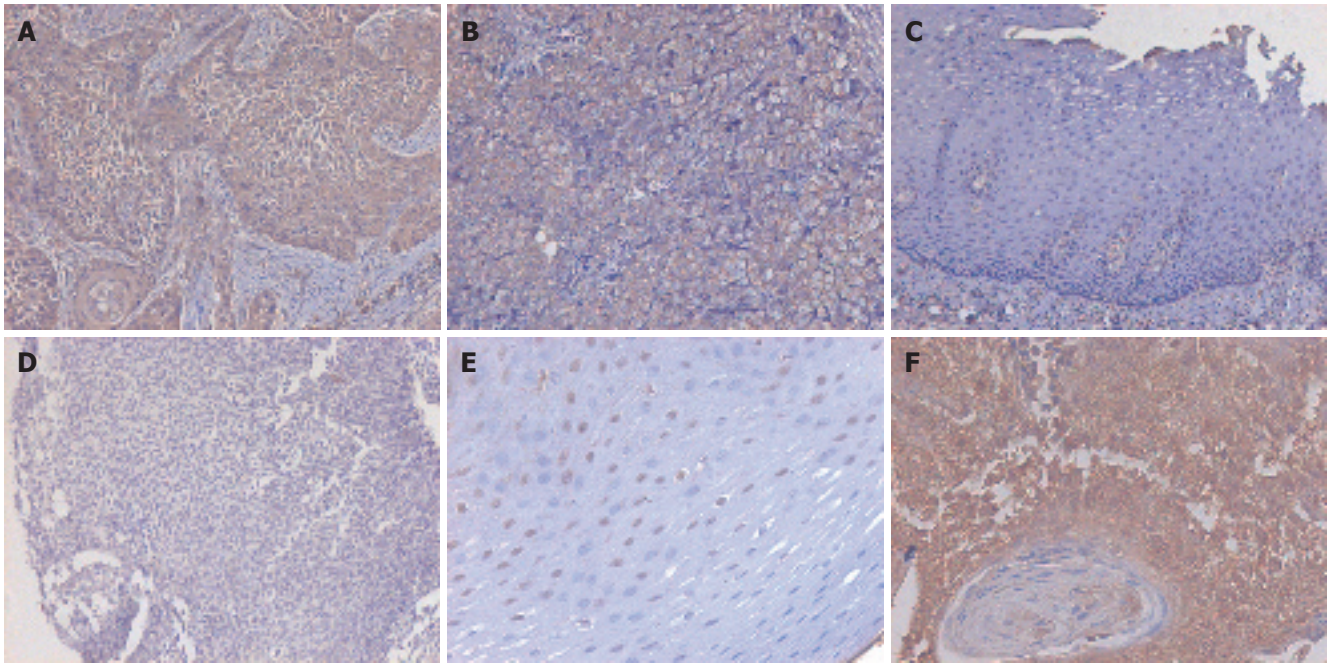

Figure 1 Immunohistochemical analysis of Elk-1 in paired ESCC samples using anti-Elk-1 antibody (1:100) showing diffuse and strong staining in cytoplasm of esophageal cancer epithelial cells well-differentiated tumor (**A**), moderately-differentiated tumor (**B**), sporadic and weak staining in the cytoplasm of normal epithelial cells (**C**), negative control designed using PBS instead of primary antibody (**D**), strong staining in nuclei of normal epithelial cells (**E**) (**A-E** × 100), and in cytoplasm of well-differentiated esophageal cancer epithelial cells (**F**) (× 200).

RESULTS

Expression of Elk-1 in esophageal squamous cell carcinoma

Positive Elk-1 expression showed brown staining signals in ESCC cytoplasm and nuclei, reduced or negative expression of Elk-1 was found in normal squamous epithelium, with only a small number of expressions in cell membranes. The increased expression rate of Elk-1 in 107 esophageal cancer patients was 78.5% (84/107) compared to that in the matched normal tissue. A significant positive correlation was found in Elk-1 expression between esophageal carcinoma tissue and paired normal squamous epithelium ($P < 0.01$). The increased expression rate of Elk-1 was 80.4% (86/107) (Figure 1).

Relationship between Elk-1 expression and clinicopathologic variables in esophageal squamous cell carcinoma

The expression of Elk-1 had no obvious correlation with the degree of differentiation of esophageal squamous cell carcinoma. The increased expression was found in 7/8 well-differentiated, 60/74 moderately-differentiated and 19/25 poorly-differentiated tumors, respectively, ($P > 0.05$). In addition, no significant correlation was found among Elk-1 expression, lymph node metastasis and depth of invasion (Table 2).

Table 2 Relationship between clinicopathologic parameters and expression of Elk-1

Type	Case	Elk-1		*P*
		Preserved	Increased	
Degree of differentiation				
Well-differentiated	8	1	7	> 0.05
Moderately-differentiated	74	14	60	
Poorly-differentiated	25	6	19	
Depth of invasion				
Mucous layer (T1)	6	1	5	> 0.05
Muscular layer (T2)	39	6	33	
Full-thickness (T3)	62	14	48	
Lymph node metastases				
Positive	35	5	30	> 0.05
Negative	72	16	56	

DISCUSSION

Regulations of cell growth are dependent on a number of gene families including proto-oncogene, growth factor, growth factor receptor and immediate early transcription factor gene. The first member of Ets gene family was discovered a decade ago by studying avian erythroblastosis virus, E twenty six (E26). Subsequently, a series of cellular Ets genes were isolated (Ets-1, Ets-2, Erg, Elk-1, Sap-1, PEA-3, PU.1, Fli-1 *etc.*)[13]. The Elk-1 gene is localized on human chromosomes Xp11.2-p11.1 and 14q32[14,15]. Elk-1 was first discovered in a fraction of HeLa cell nuclear extract that forms ternary complexes with SRF on the c-*fos* SRE. This novel component is called p62 due to its 62 kDa molecular mass[16]. Elk-1, Sap-1 and Sap-2/Net comprise the TCF subfamily of Ets-domain transcription factors. The TCF transcription factors play an important role in transducing extracellular signals into a nuclear response by acting as targets for the mitogen-activated protein kinase signaling pathways[4,5,17]. In addition to a N-terminal DNA-binding domain, Elk-1 contains a "B box" mediating its interaction with SRF, a "C domain" acting as a transcriptional activation domain, two repression domains,

and two domains that act as docking sites for multiple mitogen-activated protein kinases, including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38[4,18,19]. The ERK cascade responds to growth factors and mitogens, whereas the JNK and p38 cascades are triggered by cytokines and stress.

The understanding of the molecular basis of tumor development has progressed dramatically in the last two decades. Since tumor is essentially a genetic disease, it is important to demonstrate what these oncogenes are and how they work in carcinogenesis. Identifying the genetic differences between normal and tumor cells or tissues will help discover the genes that directly cause tumor or are associated with tumorigenesis and provide novel markers for early detection and appropriate therapy.

Elk-1 is thought to impact neuronal differentiation[20], cell proliferation[4], tumorigenesis[21], and apoptosis[22]. Elk-1 plays a role in the neuronal expression of immediate-early genes like c-*fos* in the brain[23]. Elk-1 functions as a nuclear transcriptional activator via its association with SRF on serum response elements present in the promoters of many immediate-early genes, such as c-*fos*, *egr-1*, *egr-2*, *pip92*, and *nurr77*[17]. In addition to its regulation of growth-responsive genes, Elk-1 has been shown to play a role in regulating differentiation of smooth muscle, skeletal muscle, and neuronal cells[24-26]. Recently the SRF gene has also been identified as a target for Elk-1, thereby providing a positive-feedback loop where Elk-1 activation leads to enhanced expression of its partner protein, SRF[27]. Although the role of Elk-1 has been extensively studied, little information is available concerning its involvement in esophageal epithelia. In our study, we first investigated immunohistochemically the expression of Elk-1 protein in paired ESCC by TMA. The results of IHC revealed that the expression of Elk-1 was increased 78.5% (84/107) in tumor tissue compared to that in corresponding normal tissue. A significant positive correlation was found between esophageal carcinoma tissue and paired normal squamous epithelium ($P < 0.01$). Among the 107 histologically-examined esophageal squamous cell carcinomas, eight tumors were well-differentiated, 74 moderately-differentiated and 25 poorly-differentiated, suggesting that up-regulated Elk-1 expression has no difference in degree of tumor differentiation. No significant correlation was found among expression of Elk-1, degree of differentiation, lymph node metastasis and depth of invasion. Overexpression of two target genes of Elk-1 (c-*fos*, *egr*-1 mRNAs and their proteins) were found in dysplasia and esophageal squamous carcinomas, suggesting that these genes are involved in the development of esophageal carcinoma[28]. In addition to its nuclear location, Elk-1 is found throughout the cytoplasm of tumor and normal epithelial cells (Figure 1). This is in agreement with previous studies on nuclear transcription factor Elk-1 in neuronal cells[29,30].

In conclusion, Elk-1 may have alternative extranuclear functions in esophageal carcinogenesis. The mechanism of the involvement of Elk-1 in the development and progress of esophageal carcinoma remains to be further investigated.

ACKNOWLEDGMENTS

We thank Hao Li in Anhui Medical University for providing the paraffin-embedded, formalin-fixed tissues used in this work. We are grateful to Drs. Xiao-Hui Tan and You-Yong Lu in Beijing Institute for Cancer Research, School of Oncology, Peking University, for construction of TMA.

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COMMENTS

Background

Esophageal squamous cell carcinoma (ESCC) has one of the highest malignant potentials of any tumor, and is characterized by poor survival and wide geographical variation in incidence. The molecular pathology underlying the development and progression of ESCC is poorly understood. In this study, the expression of Elk-1 was immunohistochemically examined in 107 ESCCs and its relationship with clinicopathologic parameters was analyzed.

Research frontiers

The molecular mechanisms underlying the function and regulation of the three ternary complex factors (TCFs) Elk-1, Net and Sap-1 have been extensively studied and the TCFs are a paradigm for the study of transcriptional regulation in response to extracellular signalling through the mitogen-activated protein (MAP) kinase pathway. As final effectors of multiple signalling pathways and components of protein complexes play a role in immediate early promoters, they represent key elements in the complex and dynamic regulation of gene expression.

Terminology

Tissue microarrays (TMAs) are means of combining tens to hundreds of specimens of tissues onto a single slide, using all types of *in-situ* analyses including immunohistochemistry (IHC), fluorescence *in situ* hybridization (FISH), and RNA *in situ* hybridization (RNA-ISH). Potential applications include the establishment of associations between molecular changes and clinical endpoints, testing of potential therapeutic targets using tissue samples from specific cancer patients, standardization of molecular detection of targets, and rapid translation of results from cell lines and animal models to human cancer.

Peer review

The paper contributes to the mechanisms of carcinogenesis in the squamous epithelium of esophagus. The molecular study is focused on the Elk-1 transcription factor which is overexpressed in esophageal cancer. This fact is well demonstrated, showing that increased expression of transcription factor Elk-1 may play an important role in esophageal carcinogenesis.

S- Editor Wang GP **L- Editor** Wang XL **E- Editor** Bi L

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World J Gastroenterol 2006 December 28; 12(48): 7864-7868
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Colonic exclusion and combined therapy for refractory constipation

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Received: 2006-08-22 Accepted: 2006-11-24

Peng HY, Xu AZ. Colonic exclusion and combined therapy for refractory constipation. *World J Gastroenterol* 2006; 12(48): 7864-7868

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Abstract

AIM: To investigate the therapeutic effectiveness of colonic exclusion and combined therapy for refractory constipation.

METHODS: Thirty-two patients with refractory constipation were randomly divided into treatment group (n = 14) and control group (n = 18). Fourteen patients in treatment group underwent colonic exclusion and end-to-side colorectal anastomosis. Eighteen patients in control group received subtotal colectomy and end-to-end colorectal anastomosis. The therapeutic effects of the operations were assessed by comparing the surgical time, incision length, volume of blood losses, hospital stay, recovery rate and complication incidence. All patients received long-term follow-up.

RESULTS: All operations were successful and patients recovered fully after the operations. In comparison of treatment group and control group, the surgical time (h), incision length (cm), volume of blood losses (mL), hospital stay (d) were 87 ± 16 min *vs* 194 ± 23 min (t = 9.85), 10.4 ± 0.5 cm *vs* 21.2 ± 1.8 cm (t = 14.26), 79.5 ± 31.3 mL *vs* 286.3 ± 49.2 mL (t = 17.24), and 11.8 ± 2.4 d *vs* 18.6 ± 2.6 d (t = 6.91), respectively (P < 0.001 for all). The recovery rate and complication incidence were 85.7% *vs* 88.9% (P = 0.14 > 0.05), 21.4% *vs* 33.3% (P = 0.73 > 0.05), respectively.

CONCLUSION: Colonic exclusion has better therapeutic efficacy on refractory constipation. It has many advantages such as shorter surgical time, smaller incision, fewer blood losses and shorter hospital stay.

Key words: Constipation; Colonic exclusion; Subtotal colectomy; Quality of life; Follow-up

INTRODUCTION

Refractory constipation is a common clinical symptom. Because it is very obstinate and its etiological factors are unclear, it is difficult for medical workers to treat the patients. The problem of difficult defecation usually cannot be solved with drug treatment. According to our experience, surgical treatment is suggested for the patients who are unresponsive to cathartics or who need to ingest exceeding cathartics to evacuate their bowels. There are many conventional surgical methods, such as total colectomy, subtotal colectomy, hemicolectomy, *etc*. However, the operation time of these surgical methods is too long. Besides, these surgical methods with big trauma will give rise to many postoperative complications and negatively affect the quality of the patients' lives. Moreover, the very aged patients cannot bear these operations and their treatment is far from satisfactory[1]. Since 1998 we have adopted colonic exclusion with colorectal anastomosis and treated 14 patients of refractory constipation. All of them were satisfied with the therapeutic effects. The quality of patients' lives had been improved significantly after operations. Our clinical practice demonstrates that colonic exclusion is a safe and feasible operation. It has good therapeutic effects, shorter surgical time, and lower complication incidences.

MATERIALS AND METHODS

General data

Thirty-two patients were diagnosed as refractory constipation between January 1998 and April 2006. These patients received surgical intervention after ineffective medical treatment. They were divided into two groups randomly. There were two males and twelve females in treatment group (n = 14). Their ages ranged from 31 to 77 years with a mean age of 45. Their courses of disease ranged from three to thirty years with 12 years on average. Among them, ten patients had rectocele, and eight patients had rectal prolapse. Five males and thirteen females entered control group (n = 18). Their ages ranged from 28 to 75 years with a mean age of 51. Their courses of

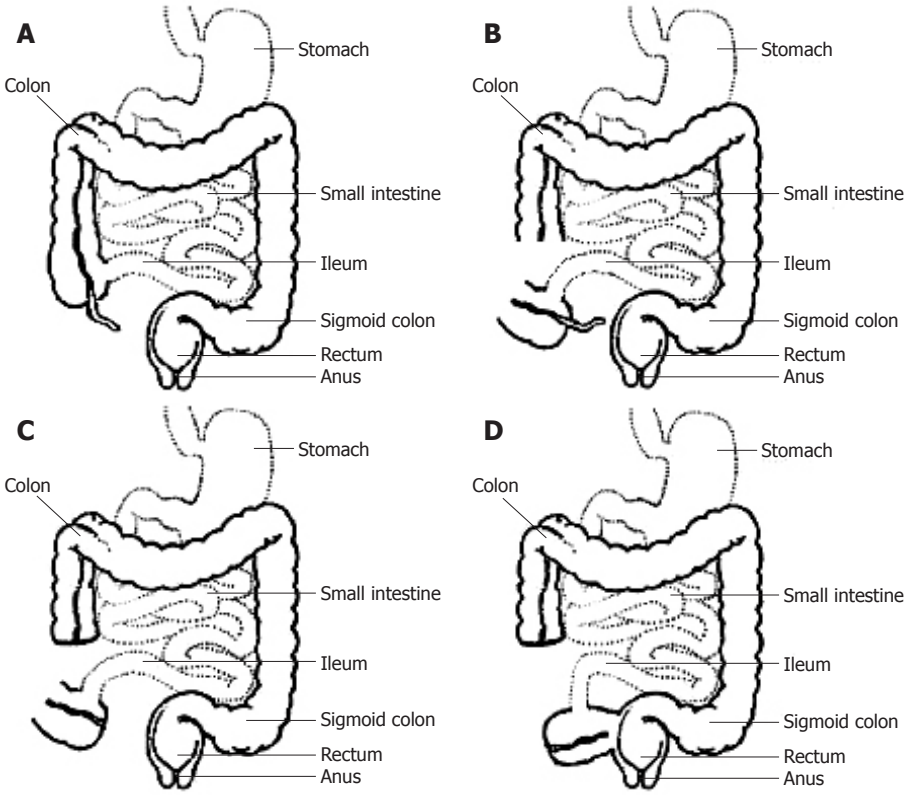

Figure 1 Surgical procedures of colonic exclusion. **A**: Normal gastrointestinal structure in human body; **B**: Transect the ascending colon at the chosen level with good blood supply; **C**: Close the distal colon by U-shape sutures and sever the vermiform appendix; **D**: Lay the distal colon in abdominal cavity. The proximal ascending is made end-to-side anastomosis with rectum.

disease ranged from four to thirty-five years with 13 on average. Eleven patients had rectocele among them. There were no statistical differences in age and courses of disease between the two groups.

Clinical symptoms

All patients had difficult defecation for three or more than three years. Some serious cases had difficult defecation beyond thirty years. They had been ingesting cathartics or other medicine for a long time. Patients usually complained of headache, general malaise, decreased appetite, abdominal pain, abdominal distension, straining at stooling, incomplete evacuation, or a need for digital manipulation to defecate. The frequency of defecation was about once four to eight days.

Diagnostic criteria

Diagnostic criteria included (1) The time of difficult defecation exceeded one year; (2) The stool frequency was below three times per week for at least three months in one year. Patients had difficult defecation accompanied by abdominal pain and abdominal distension. Stools hardened gradually to form hard feces; (3) Digital rectal examination suggested that the patient had fecal impaction accompanied by anal stenosis, hemorrhoid, and rectal prolapse; (4) Gastrointestinal transit test showed that colonic transit became slower. Rectocele and long-winded sigmoid colon were confirmed with defecography; (5) Gastrointestinal organic diseases were excluded by electronic colonoscopy.

Surgical methods

Patients in treatment group were in a lithotomy position after general anesthesia. We adopted median abdominal incision around the umbilicus. After entering the abdominal cavity, the peritoneal reflexion was severed, and the anterior wall of the rectum was dissected until the rectocele was exposed. We sewed three or four needles with 3-0 absorbable suture perpendicular to the longitudinal axis of the rectum, and subsequently sewed two needles along the longitudinal axis of the rectum. The needles should penetrate the serous membrane and muscular layer so as to restore the anterior wall of the rectum. Cautions must be taken not to penetrate the mucous membrane at the same time. Freeing of distal ileum, ileocecal junction and partial ascending colon allowed us to obtain enough slack proximal colon, which guaranteed a tension-free anastomosis. Colonic blood supply was carefully examined about 4 to 6 cm away from the ileocecal junction, and the ascending colon was transected at the chosen level with good blood supply. The distal colon was closed by U-shaped sutures to lay the indwelling colon in the abdominal cavity. Vermiform appendix was severed from the cecum by a conventional method. The proximal ascending colon and cecum were moved to the pelvic cavity in anticlockwise, and then end-to-side colorectal anastomosis was performed with stapler under peritoneal reflexion. Long-winded sigmoid colon was resected simultaneously. The rectum was raised and fixed with the lateral side of pelvic peritoneum in order to suspend the rectum. The basement of pelvic cavity was reestablished and the posterior peritoneal space was closed. Finally the abdominal cavity was closed after checking surgical instruments and gauzes. It was unnecessary to place drainage tubes in the abdominal cavity to prevent postoperative adhesion (Figure 1).

The patients in control group had median abdominal incision. After entering the abdominal cavity, peritoneal

Table 1 Surgical therapeutic efficacy for 32 refractory constipation patients

Group	Cases (*n*)	Surgical time (min)	Incision length (cm)	Blood loss (mL)	Hospital stay (d)	Recovery (%)	Improvement (%)	Complication (%)
Treatment group	14	87 ± 16	10.4 ± 0.5	79.5 ± 31.3	11.8 ± 2.4	12 (85.7)	2 (14.3)	3 (21.4)
Control group	18	194 ± 23	21.2 ± 1.8	286.3 ± 49.2	18.6 ± 2.6	16 (88.9)	2 (11.1)	6 (33.3)
t		9.85	14.26	17.24	6.91	*P* = 0.14	*P* = 0.14	*P* = 0.73
P		< 0.001	< 0.001	< 0.001	< 0.001	> 0.05	> 0.05	> 0.05

reflexion was severed. The anterior wall of the rectum was dissected until the rectocele was exposed. Then the rectocele was restored as above. The whole colon was freed. Vermiform appendix was severed. Subsequently the colon from the ascending colon, which was about 4 to 6 cm away from ileocecal valve, to sigmoid colon was resected subtotally. The proximal ascending colon and cecum were moved to the pelvic cavity in anticlockwise, and then the proximal ascending colon was end-to-end anastomosed with the rectum. The abdominal cavity was closed after closure of the posterior peritoneal space. We also did not place drainage tubes in the abdominal cavity.

Statistical analysis

All data are expressed as mean ± SD. They were entered into SPSS 12.0 statistical package. Statistical comparison was done with group *t*-test and Fisher exact probabilities in 2 × 2 table. A *P* value less than 0.05 was considered statistically significant.

RESULTS

Surgical results

The surgical time (h), incision length (cm), volume of blood losses (ml), hospital stay (d) were 87 ± 16 min, 10.4 ± 0.5 cm, 79.5 ± 31.3 mL, 11.8 ± 2.4 d in treatment group, and 194 ± 23 min, 21.2 ± 1.8 cm, 286.3 ± 49.2 mL, 18.6 ± 2.6 d in control group, respectively ($P < 0.001$) (Table 1). The treatment group had several advantages such as shorter surgical time, smaller incision, less blood losses and shorter hospital stay. Compared with the control group, colonic exclusion could reduce local trauma for many patients.

Criteria for therapeutic effect

(1) Recovery: Constipation and relevant symptoms disappeared. Auxiliary examinations showed that associated manifestations disappeared; (2) Improvement: Constipation and relevant symptoms were relieved. Auxiliary examinations showed that major relevant symptoms disappeared; (3) Inefficacy: Constipation and relevant symptoms were not obviously improved. Auxiliary examinations showed that constipation still existed.

Postoperative effects

According to the above criteria, twelve patients recovered (12/14, 85.7%) and two patients improved (2/14, 14.3%) in treatment group. In control group sixteen patients recovered (16/18, 88.9%) and two patients improved (2/18, 11.1%).

Postoperative complications

In treatment group, adhesive ileus occurred in one patient (relieved after expectant treatment later). Another patient developed grease liquefaction of incision. Acute pancreatitis occurred in one patient. Complication incidence was 21.4%. In control group, adhesive ileus occurred in three patients. The infection of incisional wound occurred in two patients. One patient developed adhesive stenosis of the ureter. Complication incidence was 33.3%. There were no death case, no postoperative stomal leak or anastomotic stricture in either groups.

Long-term follow-up

All patients were followed for six to thirty-eight months. The follow-up rate was 100%. Most patients recovered very well after operations. Their constipation disappeared and the quality of their lives improved. One patient in treatment group still had abdominal distention. She was diagnosed as endometriosis later. And the indwelling colon was resected in another hospital. The other patients did not have any abdominal pain or abdominal distention. They evacuated their bowels one to three times every day. Their stools were almost forming. Three patients in control group still had slight abdominal distention. They evacuated their bowels one to four times every day. The shape of their stools was pasty.

DISCUSSION

Over the recent years in China, along with the improvement of the economy, quickening up of the life pace, change of the structure of foods and drinks, as well as aging of the population, the incidence of refractory constipation rises year by year. It has become one of the common diseases that affect the quality of people's lives. Refractory constipation is usually classified into three types: slow transit constipation (STC), outlet obstructive constipation (OOC), mixed constipation (MC). Among them, mixed constipation is the most commonly seen in clinics.

Etiological factors

The etiological factors of refractory constipation are very complicated and largely unclear; however, some of the etiological factors have been certain[2,3]. (1) Abnormalities in the enteric nervous system; (2) Abnormalities of extrinsic nerves; (3) Smooth muscle abnormalities; (4) Interstitial cell of Cajal dysfunction; (5) Structural abnormalities of the rectum and anus: such as rectal prolapse, rectocele, hemorrhoid; (6) Endocrine and metabolic conditions;

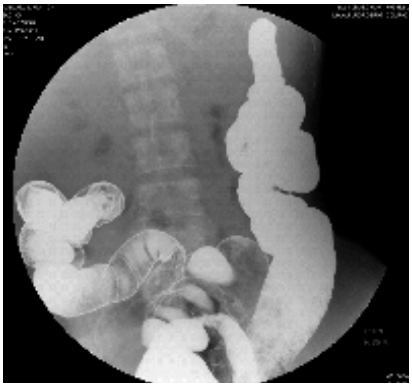

Figure 2 The indwelling colon didn't inflate and anastomotic stoma transmitted normally.

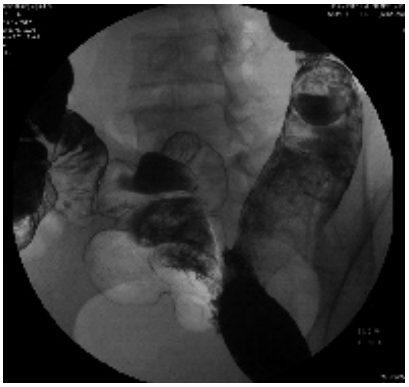

Figure 3 After twenty-four hours barium in the indwelling colon was evacuated completely.

(7) Drugs; (8) Psychogenic conditions. Understanding of the etiological factors will help us make correct treatment plans for the disease. Effective combined therapy can lead to better therapeutic efficacy.

Surgical indications

Refractory constipation is not a fatal disease. We suggest expectant treatment for the constipated patients with a short course of disease and light pathogenetic condition. Constipated patients who have serious clinical symptoms, poor quality of life and a strong desire for surgery, however, should be considered to take colonic exclusion, except for the aged patients. According to our clinical observation and experience over the years, there are the following surgical indications for colonic exclusion: (1) Patients have serious difficult defecation for at least three years. The stool frequency is below three times every week. (2) Drug treatment for at least half a year is confirmed ineffective or patients have to ingest exceeding cathartics for a long time. (3) Gastrointestinal transit test shows that colonic transit becomes slower, while the stomach and small bowel transit are normal. Accompanying outlet obstructive diseases are confirmed by preoperative defecography. (4) Constipation-predominant irritable bowel syndrome and constipations which are caused by drugs must be excluded. (5) The patient has no serious mental disorder and she (he) has a strong desire for operation. Surgeons should be cautious of surgical treatment for refractory constipation and handle surgical indications strictly. Patients should undergo preoperative psychological tests and clinical examinations of colorectal function. After that, individualized therapeutic strategies and surgical schema are made.

Variations of colonic propagated activity before and after colon exclusion

The phenomenon of slow colonic transit more or less exists in patients with refractory constipation. We used gastrointestinal transit test to detect gastrointestinal function of 14 patients in treatment group before operation. Forty-eight hours later the photographs showed that baric markers were still stagnated in transverse colon or sigmoid colon in 12 patients. Seventy-two hours later the photographs displayed retention of baric markers in sigmoid colon or rectum in nine patients. Bassotti *et al*[4] studied colonic propulsive activity in constipated patients and found colonic dysfunction. Both colonic contraction amplitude and high-amplitude propagated contractions were significantly decreased, which might be the immediate cause of colonic inertia. We examined nine patients in treatment group with barium enema after colon exclusion a month later. The intestine did not inflate and the anastomotic stoma transmitted normally (Figure 2). Three patients had barium reflux into the indwelling colon, but it was evacuated completely 24 h later (Figure 3). Hereby it demonstrated that the indwelling colon still had itself movement. It did not lose its peristalsis under the condition of disuse. However, it is uncertain whether it has any practical clinical significance to lay the colon with neuromuscular diseases in the abdominal cavity. By now, there is no definite conclusion about the long-term influence of the indwelling colon on human bodies.

Characteristics and clinical application perspectives of colon exclusion

Arbuthnot first adopted total colectomy with ileorectal anastomosis to treat slow transit constipation in 1908. Afterwards people gradually adopted total colectomy with caecorectal anastomosis. These surgical methods could relieve constipation symptoms in most patients with chronic idiopathic constipation; however, they were associated with a considerable morbidity and were less effective in resolving symptoms of abdominal pain and bloating[5]. Based on the result of gastrointestinal transit test, people adopted hemicolectomy to treat constipated patients. They only resected some segmental long-winded colon. Hemicolectomy could lead to fewer postoperative complications and a faster recovery[6], but the postoperative recurrence rate was very high[7]. This is because the excisional range of the pathological colon is not large enough. After operation the residual colon transmits slower, which will lead to recurrence of constipation. On the other hand, subtotal colectomy is a popular operation to treat refractory constipation. It seldom leads to serious diarrhea for the remaining of ileocecal valve. However, it still has some disadvantages, such as big trauma, long surgical time, delayed recovery, many complications. Besides, patients' postoperative quality of life is not so ideal[8]. We adopted colonic exclusion with colorectal anastomosis and neoplasty for symptomatic rectocele to treat constipated patients. The clinical results were satisfactory. Patients recovered excellently after operations. And there were no serious postoperative complications among them. Compared with the control group, it had

smaller incision, fewer blood losses, shorter operational time and shorter hospital stay.

In conclusion, colonic exclusion has such advantages as: (1) less trauma, fast recovery; (2) simplified operation, shorter surgical process; (3) preservation of some bowels with normal function; (4) higher clinical cure rate; (5) avoidance of serious postoperative complications. For most constipated patients, colonic exclusion is the best surgical method because it is convenient, economical, less trauma, and less painful. It is especially indicated for aged constipated patients whose surgical endurances are not so good. We assume that it has wide application value.

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COMMENTS

Background

The incidence of refractory constipation is very high in modern people, especially in aged people and middle-aged females. Some constipated patients have to be treated by surgery, while conventional surgical methods have big trauma. We recommend a new surgical method to treat refractory constipation. It is convenient, economical, less trauma, and less painful.

Research frontiers

Surgical methods to treat refractory constipation are improving. Recently people pay more attention to microinvasive operation. Laparoscopic operations instead of conventional surgical methods are used to treat refractory constipation.

Innovations and breakthroughs

Colonic exclusion is a technical innovation in treating refractory constipation. Compared with conventional surgical methods, it doesn't need to sever or resect too much colon and it retains some bowels with normal function. Rectocele is restored and the basement of pelvic cavity is reestablished. Therefore, it has less trauma and patients recover faster after operation.

Applications

Colonic exclusion has better therapeutic efficacy for refractory constipation. It has many advantages such as smaller incision, fewer blood losses, less adhesive ileus, shorter surgical time and shorter hospital stay. It is especially indicated for aged constipated patients, whose surgical endurances are not so good. It has wide application prospect in clinical practice.

Terminology

Colonic exclusion: It is a surgical method that needn't to resect the colon. The ascending colon is transected. Then the distal colon is closed and the proximal colon is end-to-side anastomosed with the rectum. An indwelling colon is laid in the abdominal cavity.

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World J Gastroenterol 2006 December 28; 12(48): 7869-7873
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

CT diagnosis of 52 patients with lymphoma in abdominal lymph nodes

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Received: 2006-09-28 Accepted: 2006-11-27

Abstract

AIM: To assess CT manifestations and its diagnostic value for lymphoma in the abdominal lymph nodes (LALN).

METHODS: CT findings in 52 cases of LALN proved by surgery or biopsy, including Hodgkin's disease (HD) in 16 cases and non-Hodgkin's lymphoma (NHL) in 36 cases, were retrospectively analyzed.

RESULTS: (1) CT manifestations based on distribution of the lesions of LALN: Solitary mass type was found in 10 cases, including solitary, round, uniform-density, enlarged lymph nodes in 3 cases; and multiple, enlarged lymph nodes fusing into singular lobular mass in 7 cases. Thirty-four cases of multiple-nodular type showed multiple, round, enlarged lymph nodes with uniform density and clear margins. Vessels-embedded signs, including mesenteric vessels, renal vessels, abdominal aorta or inferior vena cava, were seen in 6 cases, and duodenum-embedded signs were seen in 2 cases. Eight cases of diffuse type showed characteristic "cobblestone signs". (2) CT manifestations correlated with pathological type: CT manifestations of 12 cases of HD were different from those of 40 cases of NHL in distribution, size, quantity and fused lesion of enlarged lymph nodes. (3) Twenty-eight cases of 52 patients were accompanied with extra-nodal lymphoma in the abdomen, especially gastrointestinal lymphoma, which had characteristic CT findings. (4) In follow-up examinations, CT images showed uniform, heterogeneous or rim enhancement in 15 cases, and occasional calcifications accompanied with reduction of the lesion size and quantity in 12 cases, whereas the lesions disappeared in 3 cases after treatment.

CONCLUSION: CT images show many characteristic manifestations valuable for qualitative diagnosis of LALN, and it is also helpful for pathological classification of LALN and therapeutic evaluation in follow-up of patients.

Key words: Lymphoma; Lymph nodes; Computed tomography; Diagnosis; Classification

Yu RS, Zhang WM, Liu YQ. CT diagnosis of 52 patients with lymphoma in abdominal lymph nodes. *World J Gastroenterol* 2006; 12(48): 7869-7873

http://www.wjgnet.com/1007-9327/12/7869.asp

INTRODUCTION

Abdominal lymphoma is clinically not uncommon and lymphoma in the abdominal lymph nodes (LALN) is most frequently encountered[1]. Though it may be part of a systemic lymphoma, single onset of LALN is not rare. LALN can be divided into Hodgkin's disease (HD) and non-Hodgkin's lymphoma (NHL). HD is seldom seen in China[2-3] and mostly occurs in middle-aged and young people. The number of NHL patients is larger than that of HD with a diffuse distribution in age. In addition, AIDS patients are more susceptible to NHL[4].

CT is useful for diagnosis[1-2,5-14] and staging[15-21] of abdominal lymphomas. For evaluation of lymph node involvement, the sensitivity of contrast-enhanced CT is 88%, and specificity is 86%. For evaluation of organ involvement, the sensitivity of contrast-enhanced CT is 50%, and specificity is 90%[15]. To our knowledge, however, CT classification of LALN has not been reported in previous documents. We reviewed retrospectively CT findings of 52 patients with LALN confirmed by pathology and histology from 2000 to 2005 in our hospital, and analyzed its value in diagnosis, pathological classification and therapeutic assessment of LALN.

MATERIALS AND METHODS

Subjects

Of the 52 LALN patients, 29 were males and 23 females, with age ranging from 18 to 77 years (mean, 43.4 years), including Hodgkin's disease (HD, age range 18-59 years, mean 34.6 years) in 12 cases and non-Hodgkin's lymphoma (NHL, age range 20-77 years, mean 47.3 years) in 40 cases. The diagnosis was confirmed by surgery (20 cases) and lymph node biopsy (32 cases) including peripheral

lymph nodes (19 cases) and abdominal lymph nodes (13 cases). The duration of symptoms ranged from 1 to 18 mo. All the patients had evident improvement in clinical symptoms and signs, and reduction in size of the lesions or disappearance of the lesions after chemotherapy and radiotherapy.

CT scanning

Twenty patients were performed with a Somatom HiQ CT machine (Siemens Medical Systems, Forchheim, Germany) and 32 patients were performed with a 4-detector row CT machine (Volume Zoom, Siemens Medical Systems, Forchheim, Germany). All patients were in routine fasting states. Some patients were given 1000-1500 mL of diluted iodinated contrast medium (10 g/L meglumine diatrizoate) orally 60-90 min before scanning. Scan scope ranged from the dome of diaphragm to symphysis pubica. All patients were examined with plain scanning at first and then examined using Ultravist (Schering, Berlin, Germany) 80 mL for enhanced scanning, with a section thickness of 10 mm and internal of 10 mm (Somatom HiQ CT machine) or 1.5 mm section thickness, a pitch of 1.25, 5 mm reconstruction interval (4-detector row CT machine).

RESULTS

CT manifestations based on distribution of the lesions of LALN

Solitary mass type: Ten cases of solitary mass type of LALN were located in the retroperitoneum, mesenterium and lesser omentum. Of them, 3 cases on CT plain scanning showed solitary, round, enlarged lymph nodes of uniform-density with diameters of 3.2-5.8 cm. The lesions had rim enhancement in one case and slight homogeneous enhancement in 2 cases on enhanced CT. The other 7 cases showed multiple enlarged lymph nodes fusing into lobular masses with diameters of 7.8-18 cm and clear margins, of which 5 cases showed uniform density and 2 cases showed heterogeneous density on plain CT. Enhanced CT showed slight uniform enhancement in 4 cases, and heterogeneous enhancement in 3 cases. Of the all cases, the mesenteric vessels, abdominal aorta and inferior vena cava were embedded (vessels-embedded signs) in 3 cases (Figure 1).

Multiple nodular type: Thirty-four cases were of multiple nodular type of LALN, which referred to regional distribution of lymph nodes. CT showed multiple, round, enlarged lymph nodes with clear margins and uniform density in the abdomen (Figure 2). Most enlarged lymph nodes were over 1.0 cm in diameter with exception of 2 cases of less than 1.0 cm, which located in the mesenterium. Among the 34 cases, 29 showed slight uniform enhancement, and 5 appeared slight uniform enhancement accompanied with rim enhancement after contrast administration. Nineteen cases revealed a fusion of lesions of different degrees and 6 cases showed embedding of vessels (vessels-embedded signs), including the mesenteric vessels (3 cases) (Figure 3), renal vessels (3 cases), abdominal aorta (2 cases) and inferior vena cava (1 case), and 2 cases showed embedding of the horizontal segment of the duodenum (duodenum-embedded signs).

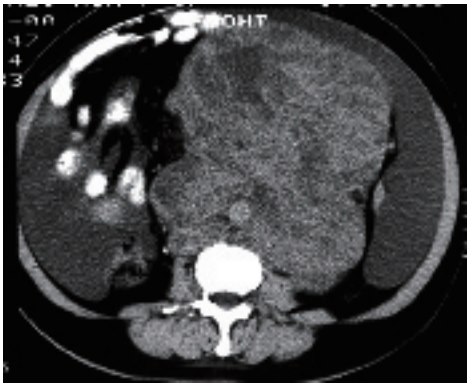

Figure 1 Solitary mass type: Enhanced CT showing multiple enlarged lymph nodes fused into uneven density, huge lobular tumors with abdominal aorta and inferior vena cava encased and ascites.

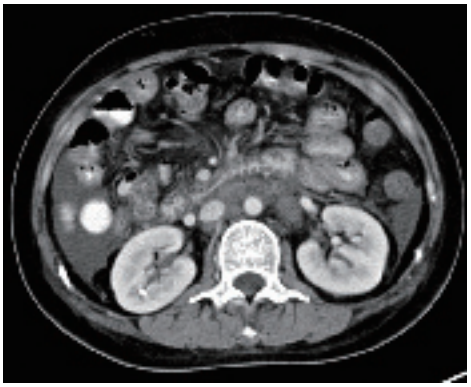

Figure 2 Multiple-nodular type: Enhanced CT showing multiple, homogeneous density, enlarged lymph nodes in retroperitoneal region with ascites.

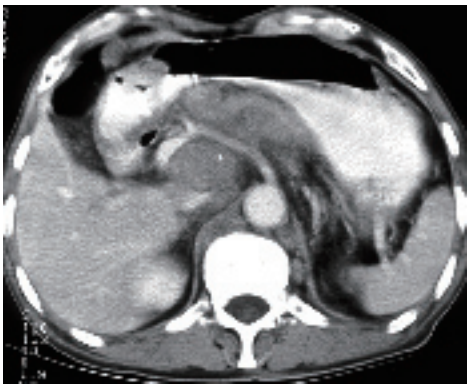

Figure 3 Multiple-nodular type: CT of portal venous phase showing mesenteric multiple enlarged lymph nodes with superior mesenteric artery encased.

Diffuse type: The diffuse type of LALN involved the whole distribution areas of abdominal lymph nodes, mainly in the mesenteric and retroperitoneal regions in eight cases. CT revealed diffuse, round, non-confluent, enlarged lymph nodes with homogeneous density and clear margins, forming the characteristic "cobblestone signs" named by us. All the enlarged lymph nodes were slightly homogeneously enhanced after contrast administration (Figure 4).

CT manifestations correlated with pathological types

HD: Twelve cases of HD included solitary mass type and diffuse type in one case each (8.3%, respectively), and multiple nodular type in 10 cases (83.4%). The enlarged lymph nodes were mainly distributed in the lesser omentum and retroperitoneal region adjacent to the abdominal aorta (Figures 2 and 5) and inferior vena cava. All the enlarged lymph nodes showed homogeneous

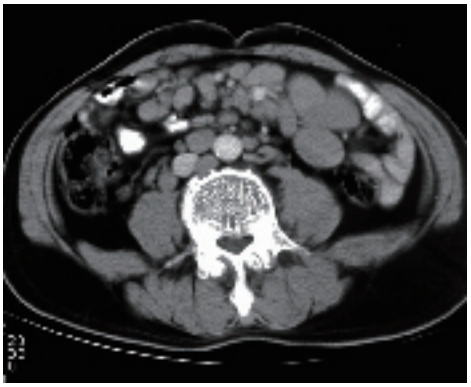

Figure 4 Diff-use type: Enhanced CT showing diffuse, clear margins, homogeneous density enlar-ged lymph nodes in the mesenteric and retroperitoneal region.

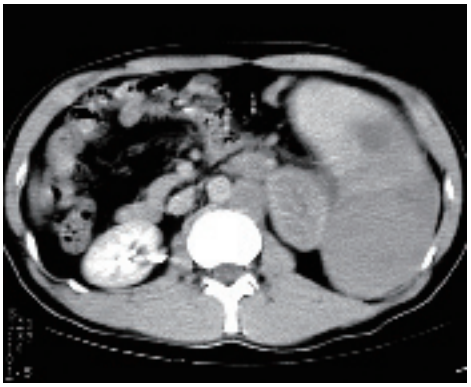

Figure 5 Enhanced CT showing multiple, homogeneous density, enlarged lymph nodes in para-abdominal aorta and multiple, low-density lesions in the spleen (splenic lymphoma).

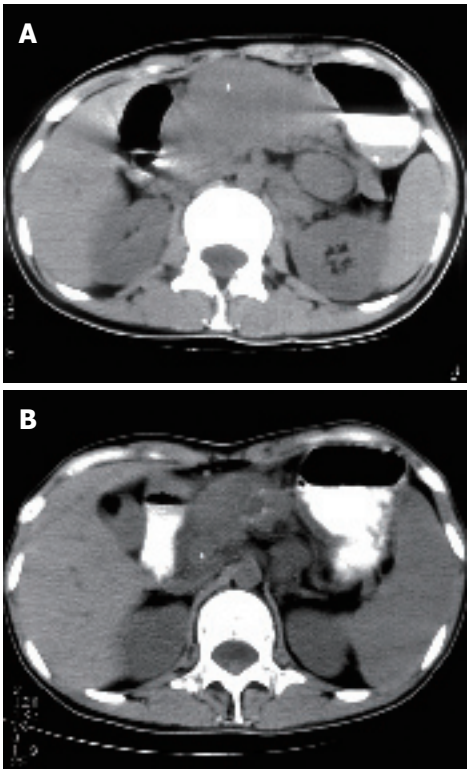

Figure 6 **A**: Plain CT showing a uniform density, slightly lobular tumor (formed by multiple enlarged lymph nodes) in the lesser omentum; **B**: The tumor having a notable shrinkage in size and flecked calcifications found within the tumor after treatment on plain CT.

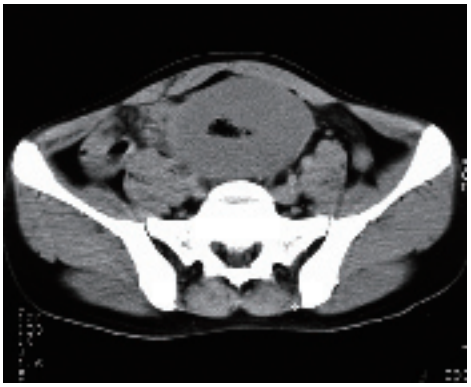

Figure 7 Enhanced CT showing notable circular thickening of intestinal wall with homogeneous density.

enhancement except one rim enhancement after contrast administration. Among the 12 cases, only one case (8.3%) involved the mesenteric lymph nodes, which showed scattered distribution of lesions and a small number of lymph node involvement with less than 2.0 cm in size. Two cases (16.7%) had confluence of lesions and 7 cases (58.3%) showed involvement of other abdominal organs (Figure 5). **NHL:** Forty cases of NHL included solitary mass type (9 cases, 22.5%), multiple-nodular type (24 cases, 60%) and diffuse type (7 cases, 17.5%) (Figure 4). All the abdominal lymph nodes were involved, including 18 cases (45%) of mesenteric involvement. Among the 40 cases, 24 (60%) showed a fusion of the enlarged lymph nodes of different degrees, appearing as lobular masses (Figures 1 and 6); 4 exhibited embedding of vessels (Figures 1 and 3) and 2 had involvement of the intestine. Twenty-one cases (52.5%) were accompanied with involvement of other abdominal organs.

CT manifestations of LALN after chemo-radiotherapy

In follow-up, CT was performed in 15 cases of LALN after chemo-radiotherapy, of which abdominal enlarged lymph nodes completely disappeared in 3 cases. The lesion size was reduced by different degrees in 12 cases, including 9 cases with homogeneous density and 3 cases with heterogeneous density, and one case with flecked calcification (Figure 6).

CT manifestations of the involvement of other abdominal organs with lymphoma

Of the 52 cases of LALN, 28 involved other abdominal organs. Among the 28 cases, intestinal tract lymphomas (13 cases) showed notable circular thickening of the intestinal wall (25-38 mm, Figure 7) in 9 cases, including 2 cases with distinct dilation of intestinal cavity and one case with coexistence of narrowing and dilation of intestinal cavity, resulting in incomplete intestinal obstruction. The other 4 cases of intestinal lymphomas developed lobular masses with uneven margins in intestinal cavity. The CT manifestations of hepatic (7 cases) and splenic (6 cases) lymphomas were solitary or multiple nodular, low-density lesions (Figure 5). Ten cases of gastric lymphomas appeared notable circular thickening of gastric wall (22-33 mm). Renal lymphomas and adrenal gland lymphomas (2 cases each) were characterized by bilateral masses of both kidneys and adrenal glands. Involvement of the peritoneum and omentum (2 cases) resulted in a large amount of ascites (Figures 1 and 2) and omental cakes.

DISCUSSION

Diagnostic value of CT for LALN

CT plays an important role in the diagnosis and staging

of abdominal lymphomas. Based on the CT features of LALN, we classified LALN into solitary mass type, multiple-nodular type and diffuse type.

Solitary mass type of LALN included singular enlarged lymph nodes and fusion of multiple enlarged lymph nodes (most cases). CT featured a huge round mass or a lobular homogeneous density mass with uniform enhancement.

Multiple-nodular type of LALN, the most frequently seen, was characterized by enlarged lymph nodes with regional distribution. Parts of enlarged lymph nodes were fused together and formed a huge mass. CT showed uniform density lesions with mild homogenous enhancement. Sometimes coexistence of mild homogenous enhancement and rim enhancement was also observed, but fully rim enhancement of all lesions was rarely seen.

All the lesions of solitary mass or multiple-nodular type had clear margins. The vessels such as mesenteric vessels, renal vessels, abdominal aorta, and inferior vena cava could be imbedded by the fused multiple enlarged lymph nodes forming the unique "vascular-imbedded signs"[5,6], which were commonly seen in multiple-nodular type. The "duodenal-imbedded signs", named by us and found in multiple-nodular type, could be induced by LALN due to rich lymphoid tissues in the horizontal segment of the duodenum, which was different from intestinal lymphomas in CT manifestations. The former was marked by narrowing of the intestinal cavity surrounded by the fusion of multiple small nodular lesions with a streak appearance and the latter was manifested as circular thickening of intestinal wall and sometimes complicated with intestinal distension. Therefore, "intestinal-imbedded signs" have an important value for the diagnosis of LALN, although this was not reported in previous documents.

The CT features of diffuse type were diffuse but non-confluent enlarged lymph nodes in the mesenteric and retroperitoneal region with uniform density and cobblestone appearance, named as "cobblestone signs". It has an important value for the diagnosis of diffuse type of LALN because it has not been seen in other diseases. Moreover, the retroperitoneal lymph nodes below the level of lumbar 2-3 vertebrae were frequently involved (HD 94%, NHL 89%, respectively), which is of great importance in the differential diagnosis with tuberculous lymph nodes [1,6].

The above classification of CT manifestations into 3 types in LALN can not only help in the radiographic diagnosis of LALN but also guide the clinical management of LALN, e.g. the scope of radiotherapy.

LALN is frequently associated with abdominal extra-nodular lymphomas, and about 54% patients had gastrointestinal tract involvement[4]. This is of significance in the diagnosis of LALN, particularly lymphomas of the gastrointestinal tract, which have a high morbidity and show characteristic circular thickening of gastrointestinal wall with notable distension of intestinal cavity on CT. More than half of the patients in this series were associated with gastrointestinal lymphomas. In addition, the association with other solid organ lymphomas such as the liver and spleen is also helpful for the diagnosis. The peritoneum and omentum are seldom involved by LALN[7-11]. There were 2 cases in this series and CT showed a large amount of ascites and omental cakes.

CT findings of HD are different from those of NHL. According to the literature and this report[6,14], we summarize the differences as follows: The enlarged lymph nodes of HD are rarely seen in the mesentery, less than 5%[14], 8.3% in this paper, with a small number of small lesions distributed dispersedly. The confluence of enlarged lymph nodes of HD is seldom seen (16.7%), which mainly occurs in multiple-nodular type (60%). NHL has a wide distribution including all sites of abdominal lymph nodes, and the mesentery is frequently involved (45%). The fusion of enlarged lymph nodes is common (60%), showing vessel-imbedded signs, intestinal-imbedded signs and cobblestone signs. NHL is seen not only in the multiple-nodular type (60%), but also in nearly all of the diffuse type and solitary type (22.5%, 17.5%, respectively).

The CT manifestations of LALN before radio-chemotherapy are different from those of LALN after treatment[22], including reductions in extent of lesions and changes in internal nodal characteristics. The CT features of LALN after treatment are characterized by the reduction in the number of enlarged lymph nodes and the shrinkage of lesions. Though most enlarged lymph nodes remain uniform density on CT, an evident increase in heterogeneous or rim enhancement of lesions may occur due to intra-nodular necrosis after treatment and calcifications of lesions may occur in individual cases[5]. The results of this series revealed that 20% lesions disappeared after radio-chemotherapy and most of these occurred in multiple-nodular type with relative concentration of enlarged lymph nodes. Therefore, CT is valuable for the evaluation of therapeutic efficacy of radio-chemotherapy and helps guide the following treatment.

Differential diagnosis of LALN

Lymph node tuberculosis: Lymph node tuberculosis may be easily confused with lymphomas and should be considered emphatically. Lesions of lymph node tuberculosis are relatively concentrated (non-hematogenous disseminated type) and the mesenteric lymph nodes are commonly involved. CT shows rim or multilocular enhancement of enlarged lymph nodes, and sometimes with intra-nodular calcifications. This disease is often associated with tuberculous peritonitis, which has characteristic signs of omental cakes, smudged mesentery signs and high-density ascites. LALN is relatively disperse and the mesenteric lymph nodes are not commonly involved in HD. CT shows homogenous enhancement of lymph node enlargement with fewer calcifications (only 0.84%) in untreated cases[12-13] and peritoneal and omental lymphomatosis is not often seen. In addition, rim enhancement of enlarged lymph nodes of LALN (including before and after radio-chemotherapy) on CT is different from that of lymph node tuberculosis. The lesions of LALN with clear margins and multilocular enhancement are not often found.

Metastatic tumor: The occurrence of abdominal lymph node metastasis usually follows the development of a

primary tumor. The enlarged lymph nodes are located near the primary lesion, showing heterogeneous or rim enhancement due to necrosis of lymph nodes, with frequent occurrence of ascites. Neither tuberculous lymph nodes nor metastatic lymphadenopathy has blood vessel-imbedded signs, intestinal-imbedded signs, or cobblestone signs.

In summary, LALN has three kinds of CT manifestations and each kind has some unique features valuable for qualitative diagnosis of LALN. CT is also helpful for the pathological classification of LALN and the therapeutic evaluation in follow-up.

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S- Editor Wang J **L- Editor** Zhu LH **E- Editor** Ma WH

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World J Gastroenterol 2006 December 28; 12(48): 7874-7877
World Journal of Gastroenterology ISSN 1007-9327

CASE REPORT

Sporadic ganglioneuromatosis of esophagogastric junction in a patient with gastro-esophageal reflux disorder and intestinal metaplasia

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Received: 2006-04-14 Accepted: 2006-11-23

Abstract

A 58-year-old female with a recurrent history of upper abdominal pain and intermittent dysphagia underwent endoscopic evaluation that demonstrated an irregular and nodular esophago-gastric (EG) junction and grade Ⅰ erosive esophagitis. Biopsies showed prominent intestinal metaplasia of Barrett's type without dysplasia, chronic inflammation and multiple aggregates of large cells within the mucosal lamina propria, some with spindle shaped nuclei. Immunohistochemistry stains for keratins AE-1/AE-3 were negative, while S-100 and NSE were positive. This, together with routine stains, was diagnostic for mucosal ganglioneuromatosis. The background of chronic inflammation with intestinal type metaplasia was consistent with long-term reflux esophagitis. No evidence of achalasia was seen. Biopsies of gastric antrum and fundus were unremarkable, without ganglioneural proliferation. Colonoscopy was unremarkable. No genetic syndromes were identified in the patient including familial adenomatous polyposis and multiple endocrine neoplasia type Ⅱb (MEN Ⅱb). Iansoprazole (Prevacid) was started by oral administration each day with partial relief of symptoms. Subsequent esophagogastroscopy repeated at 4 mo showed normal appearing EG junction. Esophageal manometry revealed a mild non-specific lower esophageal motility disorder. Mild motor dysfunction is seen with gastro-esophageal reflux disease (GERD) and we feel that the demonstration of localized ganglioneuromatosis was not likely related etiologically. In the absence of findings that might suggest neural hypertrophy, such as achalasia, the nodular mucosal irregularity seen with this instance of ganglioneuromatosis may, however, have exacerbated the patient's reflux.

Key words: Manometry; Motility; Lower esophago-gastric junction; Esophagus; Ganglioneuromatosis

Siderits R, Hanna I, Baig Z, Godyn JJ. Sporadic ganglioneuromatosis of esophagogastric junction in a patient with gastro-esophageal reflux disorder and intestinal metaplasia. *World J Gastroenterol* 2006; 12(48): 7874-7877

http://www.wjgnet.com/1007-9327/12/7874.asp

INTRODUCTION

This paper presents a solitary ganglioneuroma of the esophago-gastric junction in a 58-year old woman with a chief complaint of recurrent bouts of abdominal pain and mild dysphagia over several years and a recent onset of diarrhea. There are several primary esophageal tumors, which show neuroid differentiation, most arising from innate innervations of the esophagus.

These may be solitary or disseminated with Schwann cell and ganglion cell components involving any portion of the gastrointestinal tract. Cases may demonstrate either an exophytic polypoid or endophytic configuration and tend to arise from the neural plexus in the bowel wall. Non-neoplastic neural proliferations involving the esophagus include achalasia with inflammation and localized neural proliferation, which closely mimics ganglioneuromatosis. Neoplastic neural tumors that can involve the esophagus include ganglioneuroma, gastrointestinal autonomic nerve tumor (GAN), schwannoma, neurofibroma, granular cell tumor; and gangliocytic paraganglioma. Ganglioneuroma is a benign tumo, and the solitary variant may be cured by excision. Lesions in syndromic ganglioneuromatosis may require more extensive surgery.

CASE REPORT

Clinical presentation

The patient is a 58-year old Caucasian woman who presented with a complaint of recurrent bouts of abdominal pain, mild dysphagia over several years and recent onset of diarrhea. Previous medical history was significant for hyperlipidemia, insulin dependent diabetes mellitus, coronary artery disease, pancreatitis and diagnosed meningioma. Previous surgical history included cholecystectomy, appendectomy, cervical spine

fusion and celiac axis stenting. Medicactions prior to endoscopic biopsy of esophagogastric EG junction included the following: Atorvastatin, pioglitazone HCl, diphenhydramine, Glipizide, Pyridoxine, isosobide and dihydrocodeinone.

Endoscopic and microscopic findings

Endoscopy showed a somewhat nodular and irregular appearing EG junction with non-erosive mucosa (Figure 1). The antrum showed mild non-erosive gastritis. The pylorus and duodenum were unremarkable. Biopsies of the EG junction, antrum and duodenum were obtained. Hematoxylin and Eosin stained histologic sections from the EG junction showed aggregates of tangled fascicles of large cells mixed with Schwann like spindle cells expanding lamina propria (Figure 2). At low magnification this gave a pseudo granuloma appearance with distortion of the adjacent gland architecture. Higher magnification revealed a fibrillar cytoplasmic matrix with monomorphic bland nuclei without mitotic activity. Differential diagnosis for these features included ganglioneuroma, neurofibroma and schwannoma[1]. Immunohistochemical staining for S100 demonstrated a nerve sheath component. Focal positivity for Synaptophysin confirmed the presence of a ganglioneuronal tissue (Figure 3). The glandular epithelial component showed frequent goblet cells with no evidence of dysplasia. Chronic inflammatory cells were scattered in mucosal stroma. The biopsy diagnosis based on both HE and immunophenotypic characterization was of localized ganglioneuromatosis of EG junction within a background of mild chronic inflammation and prominent intestinal metaplasia without dysplasia.

Treatment and follow-up

Clinical correlation excluded the presence of achalasia or MEN-ⅡB. Treatment following this diagnosis of localized ganglioneuroma associated with esophagitis included prevacid 30 mg daily. Neurontin had been considered if symptoms would have progressed; however, this was not the case. A follow-up observation with repeat endoscopic evaluation at 4 mo was performed and showed mild gastritis with mild chronic inflammation in an unremarkable EG junction without ulceration or nodularity. Early recognition of symptomatology that might suggest either lower esophageal sphincter (LES) dysfunction or esophageal dysmotility was reviewed with the patient. No other family members were symptomatic or presented a history that might suggest hereditary or familial factors. Colonoscopy showed no discrete lesions.

Except for occasional bouts of mild non-specific abdominal pain and mild dysphagia, the patient remained well for a year. There was no complaint, odynophagia or nausea. Esophageal manometry was performed for persistent dysphagia at one year following the initial biopsy. This demonstrated a distal esophageal amplitude of 106 mm mercury with a duration of 3.5 s, peristaltic contraction 90% with 10% simultaneous contractions. Detailed review of the manometric data suggested a mild non-specific lower esophageal motility disorder.

In the absence of changes that might suggest neural hypertrophy, occasionally seen with achalasia, the mild motor dysfunction in this patient may be related to GERD. The demonstration of localized ganglioneuromatosis was most likely fortuitous and not etiologically related to the gastroesophageal reflux. However, the patient's insulin dependant diabetes might have affected esophageal motility possibly associated with an increased incidence of reflux.

It is most likely that the generous biopsy sampling of the nodular EG may have removed a significant portion of the lesion; therefore, it was not overtly visible on the subsequent endoscopy.

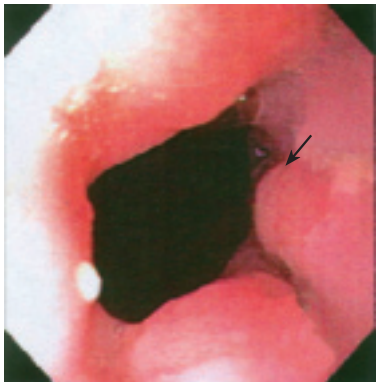

Figure 1 Initial endoscopic image of localized nodularity in esophageal mucosal surface with irregular Z-line.

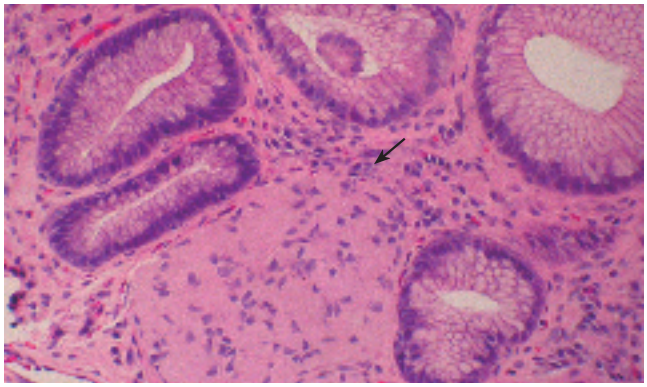

Figure 2 Hematoxylin and eosin stained tissue section of biopsy from irregular EG-Junction showing neuroid proliferation within lamina propria (× 10).

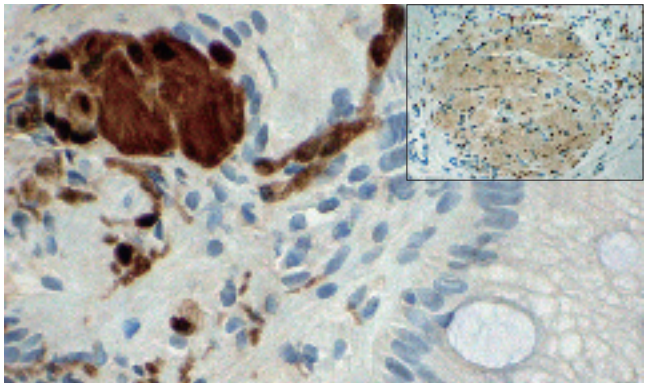

Figure 3 Focal positivity for synaptophysin (insert × 10) confirms the presence of a neuronal (ganglio) component. S100 (× 20) confirms nerve sheath-neuromatous/ neuromatosis component.

DISCUSSION

Primary esophageal tumors that show neuroid differentiation in addition to ganglioneuromata, include gastro-intestinal autonomic nerve tumor (GAN)[2,3], schwannoma[4,5] (some occasionally showing melanocytic differentiation)[6], and neurofibromas[7]. The innervation of the lower esophagus includes parasympathetic supply from the vagi and sympathetic innervation from the greater splanchnic and thoraci ganglia[8]. Histologically the neural components are seen within the muscle layer as myenteric plexus and in the submucosal neural plexus with branches entering the lamina propria[9].

Solitary or disseminated Schwann cell and ganglion cell proliferation anywhere in the gastrointestinal tract may appear as small intramucosal nodular lesions[10], exophytic polypoid lesions, or poorly demarcated transmural proliferations[11]. Ganglioneuroma, a fully differentiated tumor with no immature components[12], may occur as a solitary lesion (sporadic) or as multiple lesions called ganglioneuromatosis and may be associated with other diseases (syndromic).

Sporadic ganglioneuroma has been unknown to be associated with genetic syndromes and has been detected in patients of all ages with a mean age of 50 years. The majority of the solitary lesions are asymptomatic and, therefore, found incidentally, most frequently in the left colon[13].

Among the cases of multiple lesions, ganglioneuromatosis of an exophytic polypoid type (ganglioneuromatous polyposis) is characterized by interposition of neural proliferations with glandular components and is usually associated with familial adenomatous polyposis and multiple cutaneous lipomas. Ganglioneuromatosis of transmural proliferation type arising from the neural plexus in the bowel wall is frequently associated with other tumors, including MEN Ⅱb (medullary carcinoma thyroid, pheochromocytoma, oral-mucosal neuromas and skeletal deformities)[14], multiple ganglioneuromas and neurofibromas of the gastrointestinal tract, von Recklinghausen's disease, and neurogenic sarcoma[15]. Florid hyperplasia of submucosal or myenteric plexus is distinct for intramural ganglioneuromatosis and occurs with type Ⅰ neurofibromatosis[16]. Patients with syndromic ganglioneuromatosis present with symptoms, and the lesions are found much earlier in life, with a mean age approximately 35 years. There is no gender predominance in the incidence of this disease.

Non neoplastic neural proliferations involving the esophagus include achalasia, which is an esophageal motor disorder associated with a loss of myenteric ganglion cells with inflammation and secondary changes including neural proliferation, which closely mimics ganglioneuromatosis[17]. Neoplastic neural tumors that can involve the esophagus include ganglioneuroma, GAN, schwannoma, neurofibroma and other less prevalent forms like granular cell tumor with large eosinophilic cells[18]; gangliocytic paraganglioma showing predominantly spindle shaped cells with both ganglion and neuroendocrine features (more often seen in duodenum)[19]. Ganglioneuromatosis is visibly present throughout the gut showing predominantly spindle shaped neural proliferations with frequent ganglion type cells.

Ganglioneuroma is a benign tumor and the solitary variant may be cured by excision of the nodular lesion or complete polypectomy. Lesions in syndromic ganglioneuromatosis may require surgery, but the patient may die from the associated syndromes.

In summary, this case illustrates an instance of sporadic ganglioneuromatosis involving EG junction in a 58-year old female with a recurrent history of upper abdominal pain. Background of chronic inflammation and intestinal type metaplasia suggested that the symptoms were related to reflux esophagitis, possibly exacerbated by the nodular growth of the ganglioneuromatosis, which involved the gastro-esophageal junction. The attendant mild lower esophageal motility disorder (demonstrated by manometry) may reflect nonspecific reflux disorder. The patient was initially treated with Iansoprazole (Prevacid), which resulted in a partial relief of symptoms. This was followed by esomeprazole (Nexium) with additional relief of symptoms. The patient continued to experience mild intermittent dysphagia without odynophagia or nausea. Repeat EG endoscopy at 4 mo showed unremarkable GE junction. The importance of recognizing symptomatology indicative of lower esophageal sphincter dysfunction was reviewed with this patient with a discussion of follow-up studies over time[20]. It appears that the initial generous biopsy sampling removed the EG tumor nodularity, and this, together with the anti-acid treatment, decreased the intensity of the reflux esophagitis. Following the manometric study the patient was advised to swallow slowly with at least 30 s intervals, avoid beverages with extreme temperatures and was maintained on her current medications. We believe that although not etiologically associated, localized esophageal ganglioneuromatosis may exacerbate aspects of gastro-esophageal reflux disorder.

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S- Editor Wang GP **L- Editor** Zhu LH **E- Editor** Liu WF

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World J Gastroenterol 2006 December 28; 12(48): 7878-7883
World Journal of Gastroenterology ISSN 1007-9327

CASE REPORT

Osteoclast-like giant cell tumors of the pancreas and liver

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Received: 2006-06-12 Accepted: 2006-11-21

Abstract

Osteoclast-like giant cell tumors (OGCT) are rare abdominal tumors, which mainly occur in the pancreas. The neoplasms are composed of two distinct cell populations and frequently show an inhomogenous appearance with cystic structures. However, due to the rarity of these tumors, only very limited clinical data are available. Imaging features and sonographic appearance have hardly been characterized. Here we report on two cases of osteoclast-like giant cell tumors, one located within the pancreas, the other within the liver, in which OGCTs are extremely rare. Both patients were investigated by contrast sonography, which demonstrated a complex, partly cystic and strongly vascularized tumor within the head of the pancreas in the first patient and a large, hypervascularized neoplasm with calcifications within the liver in the second patient. The liver OGCT responded well to a combination of carboplatin, etoposide and paclitaxel. With a combination of surgical resection, radiofrequency ablation and chemotherapy, the patient's survival is currently more than 15 mo, making him the longest survivor with an OGCT of the liver to date.

Key words: Osteoclast-like giant cell tumor; Liver cancer; Pancreatic cancer; Contrast sonography

Bauditz J, Rudolph B, Wermke W. Osteoclast-like giant cell tumors of the pancreas and liver. *World J Gastroenterol* 2006; 12(48): 7878-7883

http://www.wjgnet.com/1007-9327/12/7878.asp

INTRODUCTION

Osteoclast-like giant cell tumors (OGCTs) have been described in several extraskeletal sites. Within the alimentary tract, these rare neoplasms mainly occur within the pancreas. Since the first description by Rosai in 1968[1] and further classification by Alguacil-Garcia and Weiland in 1977[2], there have been at least 38 publications describing approximately 60 occurrences of OGCTs in the pancreas[1-15], whereas only 12 liver tumors have been reported[16-27].

CASE REPORTS

Case 1

A 54-year-old man regularly visited his urologist because of prostate hyperplasia. After a renal ultrasound was suspicious of a tumor within the left kidney, computed tomography (CT) of the abdomen was performed. CT only showed a parenchyma bridge within the left kidney but surprisingly demonstrated a 7 cm large inhomogenous solid liver tumor involving segments Ⅱ and Ⅲ. The patient was then referred to our clinic. He had no clinical symptoms, no history of liver diseases and denied alcohol and tobacco consumption. His further medical history was remarkable for peripheral arterial disease, hypertension, esophageal reflux, hiatal hernia and sleep apnea.

Physical examinations revealed an obese male (173 cm, 92 kg, BMI: 31) with normal vital signs. His abdomen was soft and nontender. Serum chemistries were within the normal range except for a gamma-glutamyl transferase level of 69 IU/L (normal range < 55 IU/L) and normocytic, normochromatous anemia [hematocrit 0.38% (0.4%-0.52%)]. Serum alpha-fetoprotein, carcinoembryonic cancer antigen and cancer antigen 19-9 were normal. Serology for hepatitis A, B, and C was negative.

B-mode sonography (HDI 5000, Philips) demonstrated a well-defined, inhomogenous, cauliflower-like tumor with multiple small calcifications, causing retraction of the liver contour. Within the center of the tumor, a focal nodular hyperplasia (FNH)-like stellar scar was present (Figure 1A). Contrast-enhanced sonography by use of an Acuson Sequoia 512 (Siemens, Germany) with Sonovue® (2 mL; Bracco, Italy) using coded pulse sequences (CPS) and a low mechanic index (MI) demonstrated an inhomogenous perfused tumor with a large feeding artery heading towards the center of the tumor, radially branching to the periphery. During the capillary and arterial perfusion phase, non-perfused tumor areas indicative of necrotic areas were observed, making a focal nodular hyperplasia highly unlikely (Figure 1B). Diagnosis of haemangioma or adenoma was excluded by the spoke-like architecture of tumor arteries with centrifugal filling (Figure 1B). The portal perfusion phase was characterized by a rapid decrease of perfusion

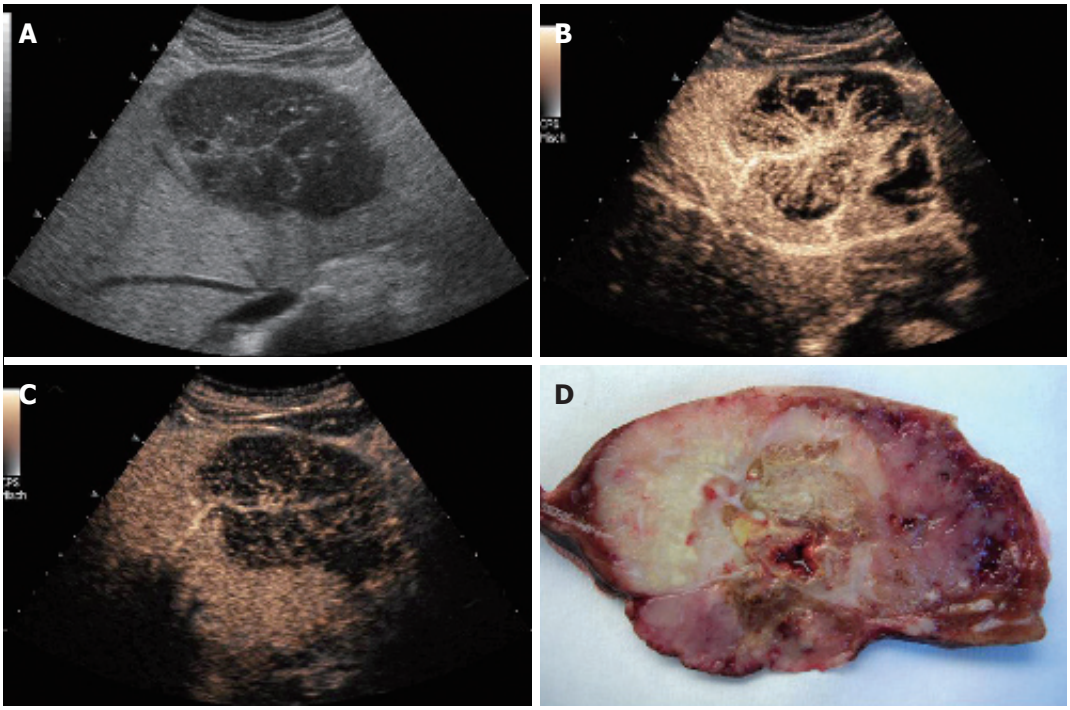

Figure 1 Patient 1: Osteoclastic giant cell tumor of the liver: **A**: B-mode sonography (HDI 5000, Philips), contrast sonography during the **B**: arterial and **C**: portal phase (Siemens); **D**: resected tumor: cut surface shows distinct tumor areas with necrotic and hemorrhagic regions.

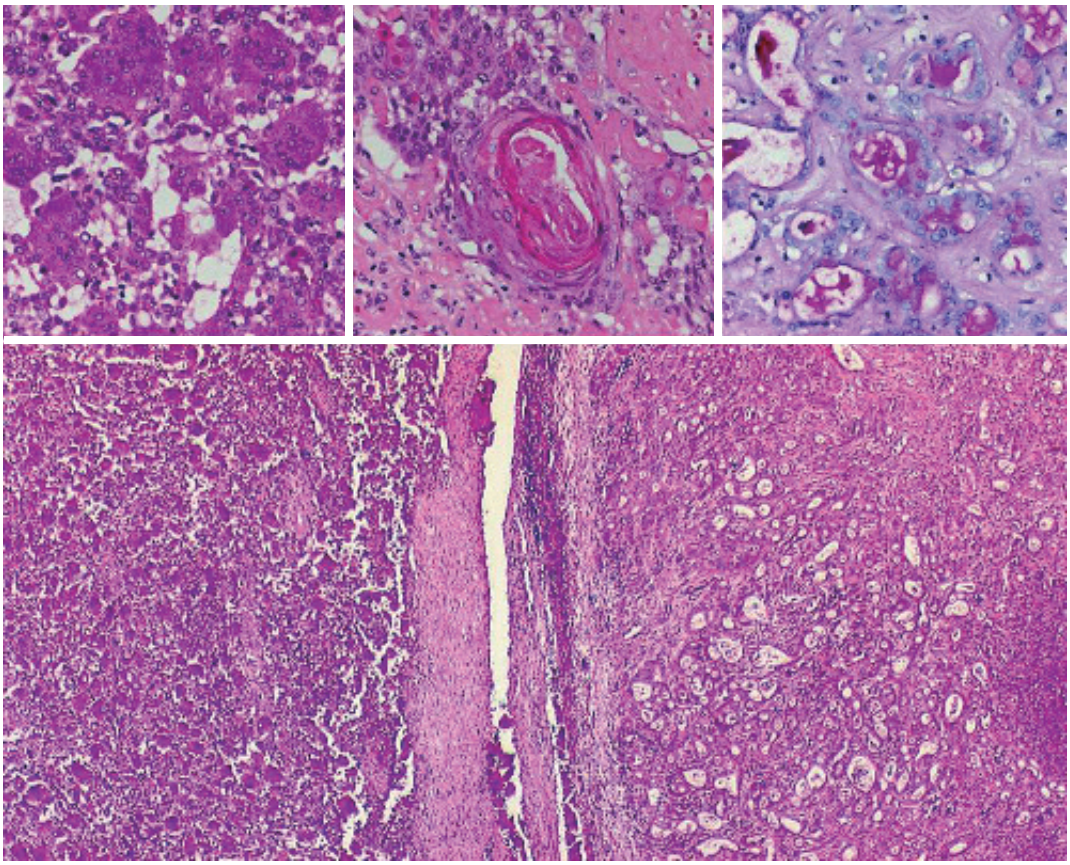

Figure 2 Histology (HE, x 20-40) of OGCT of the liver with a mixed cell population of osteoclastic giant cells and pleomorphic mononuclear cells (left), adenocarcinomatous component with mucinous inclusions (PAS, upper right) and squamous cell differentiation (middle).

within the tumor (Figure 1C), indicating absence of portal vessels and thereby, together with the finding of a strong perfusion within the arterial phase, proving a malignant neoplasm.

Biopsy of the tumor was performed, demonstrating that the neoplasm was composed of pleomorphic mononuclear cells and scattered non-neoplastic osteoclast-like giant cells (OCGCs) with usually 10-20 uniformly small nuclei. Small mononuclear cells were differently characterized by cytological atypias and showed an increased number of mitoses with a proliferation rate of 70%. Focally there was an adenocarcinomatous component with mucinous inclusions and also areas of squamous cell differentiation (Figure 2). Immunofluorescence

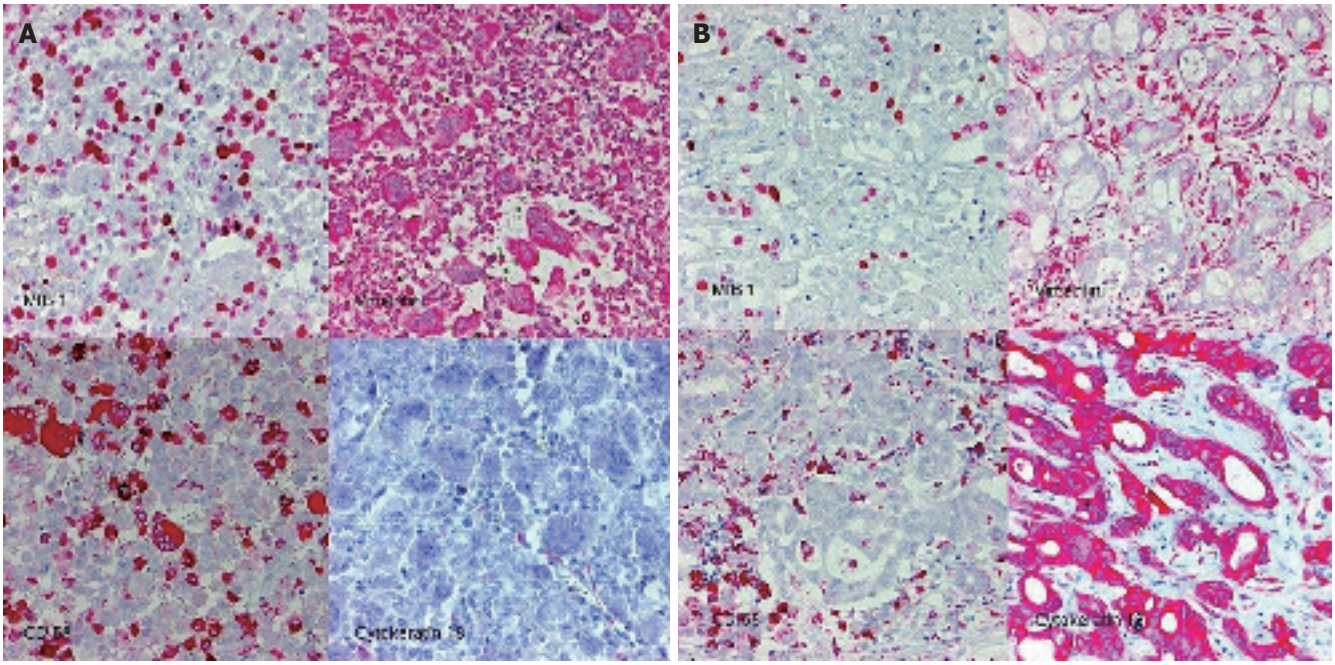

Figure 3 Immunohistology of the OGCT of the liver. Panel **A**: mixed cell population of osteoclastic giant cells and pleomorphic mononuclear cells. CD68: histio-monocytic differentiation of osteoclastic giant cells, MIB 1: proliferation of mononuclear cells. Panel **B**: adenocarcinomatous component with mucinous inclusions: CK18+, CD68/ vimentin: -.

demonstrated expression of vimentin within both OCGCs and mononuclear cells, expression of macrophage marker CD 68 within OCGC and negative staining for CK18 (Figure 3). The adenocarcinomatous tumor components demonstrated expression of CK18 but negative staining for CD 68 and vimentin (Figure 3). In conclusion, the histopathological diagnosis revealed an osteoclast-like giant cell tumor of the liver.

As extensive further diagnostic procedures including gastroscopy, colonoscopy, endoscopic ultrasound and bone scintigraphy showed no evidence of another tumor or metastases, surgical resection of liver segments Ⅱ and Ⅲ with excision of regional lymph nodes was performed. Macroscopic examination of the resected specimen revealed an irregular shaped firm mass measuring 8.5 cm with hemorrhage and necrosis (Figure 1D). All surgical margins were free of tumors. However, two lymph nodes (retroduodenal and at the hepatic artery) showed metastases of an adenocarcinoma.

As OGCT's are typically aggressive tumors with a short life-expectancy, adjuvant chemotherapy consisting of carboplatin 250 mg/m^2 (d 1), etoposide (alternating doses of 50 and 100 mg, d 1-10) and paclitaxel (175 mg/m^2, d 1) was initiated. However, 6 wk after resection, as the first chemotherapy had just been completed, a single metastasis in the right lobe of the liver was diagnosed. The metastasis was successfully treated by radiofrequency ablation. Chemotherapy was proceeded for 6 mo. After restaging revealed no evidence of active tumor disease, chemotherapy was stopped. However, 3 mo after cessation of chemotherapy, tumor recurrence (liver and peritoneal metastases) was observed and chemotherapy (carboplatin, etoposide, paclitaxel) restarted. Restaging after 6 wk revealed partial remission with size reduction of metastases of 50%. Chemotherapy is currently proceeded. The patient's survival since diagnosis has been 15 mo.

Case 2

A 49-year-old man suffered from pain in the upper abdomen since 4 mo ago, which was independent of food intake. During this time, his appetite and body weight were unchanged, however, his physical performance was reduced. Abdominal ultrasound revealed a 3 cm cystic mass in the region of the body and tail of the pancreas. The patient was then referred to our clinic for further diagnosis.

At admission he presented in fairly well condition with normal body weight (186 cm, 82 kg, BMI: 21). His abdomen was soft and nontender without evidence of ascites or signs of liver disease. Laboratory work-up showed no pathologic results except for moderate normocytic and normochromatous anemia (Hct 0.36% [0.4%-0.52%]). Neuron-specific enolase was slightly elevated (19.2 μg/L [< 15.2]), carcinoembryogenic antigen (CEA) and cancer antigen (CA) 19-9 were normal. He had stopped smoking 2 years ago and reported only moderate alcohol consumption. His past medical history was unremarkable. His family history revealed prostate carcinoma of his father.

B-mode sonography in our clinic (HDI 5000, Philips) indicated rapid enlargement of the pancreatic neoplasia, which presented as a well-defined complex lesion with several cysts and solid parts and had grown from 3 to 5 cm within 5 wk (Figure 4A). Contrast sonography by use of an Acuson Sequoia 512 with Sonovue® and CPS/low-MI technique demonstrated an intensively perfused tumor with strong vascularized margins and septs (Figure 4B), excluding a ductal pancreatic carcinoma. Strong vascularised pancreatic neoplasms have primarily to be differentiated

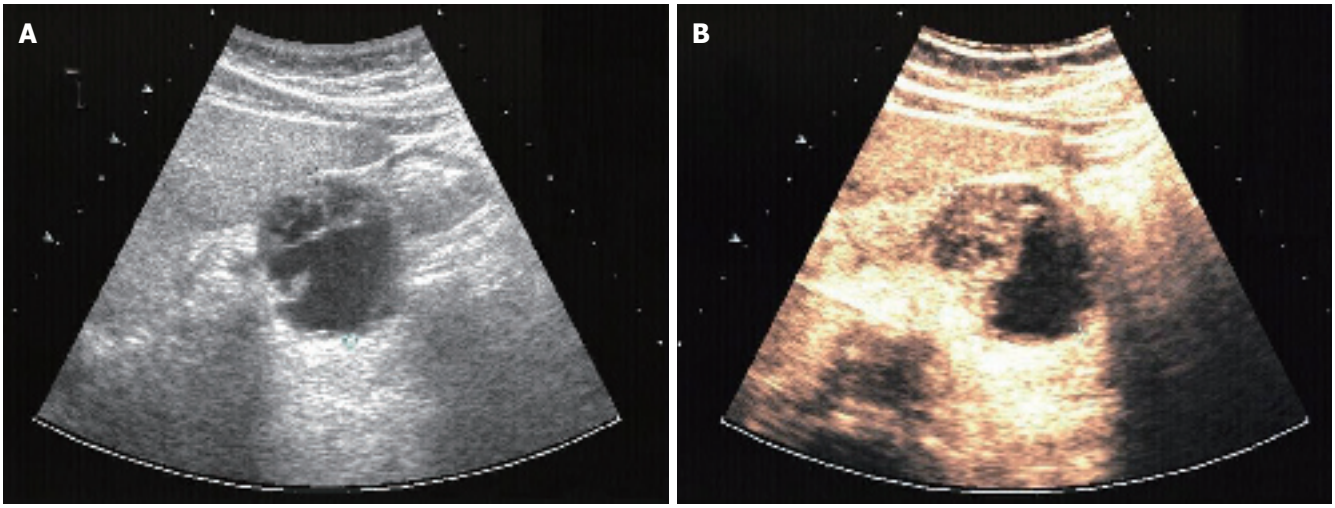

Figure 4 Patient 2: osteoclastic giant cell tumor of the pancreas. **A**: B-mode (HDI 5000, Philips) and **B**: contrast sonography (Sonovue®, low MI/CPS, Acuson Sequoia 512).

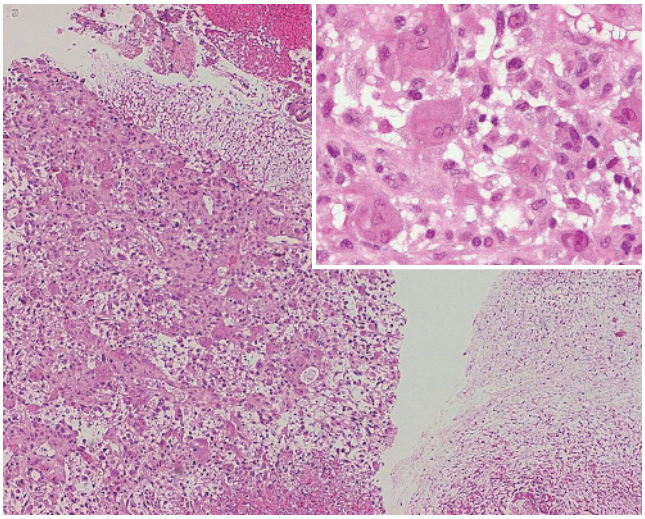

Figure 5 Histology (HE, x 20-40) of OGCT of the pancreas with a mixed cell population of osteoclastic giant cells and pleomorphic mononuclear cells (left) and myxoid tumor areas (right).

from neuroendocrine tumors of the pancreas and from cystadenomas, which may also demonstrate strong vascularisation. However, malignant diseases become apparent after liver sonography. B-mode sonography demonstrated two complex nodular structures of 1 and 3 cm. Contrast sonography showed intensively perfused lesions with necrotic areas without portal perfusion, indicating metastases.

Biopsy revealed that the neoplasm was composed of pleomorphic mononuclear cells and scattered non-neoplastic osteoclast-like giant cells (OCGCs) with multiple uniformly small nuclei. OCGC's occasionally contained phagocytosed mononuclear cells. Immunohistochemically, the OCGC's were negative for cytokeratin but positive for vimentin and CD 68. Immunohistology of the pleomorphic cells demonstrated expression of vimentin, negative staining for cytokeratin and a proliferation rate of 30% (MIB-1). In conclusion, the histopathological diagnosis indicated osteoclast-like giant cell tumor of the pancreas (Figure 5).

As surgical resection was not possible, the patient received palliative chemotherapy with cisplatin 80 mg/m^2 (d 1), etoposide (100 mg/m^2, d 1, 3, 5) and ifosfamide (2 g/m^2, d 1-3) (PEI). After completion of three cycles of PEI, partial remission with significant size reduction of liver metastases was achieved. Thirteen mo after diagnosis chemotherapy is currently proceeded.

DISCUSSION

Osteoclast-like giant cell tumors typically display an inhomogenous macroscopic appearance with cystic-liquid and necrotic areas and parenchymatous and calcified parts. Histologically, this mixed macroscopic structure corresponds to heterogeneous tissue structures of epithelial or undifferentiated tumor cells, which may contain foci of conventional adenocarcinoma, focal cartilagenous differentiation and bone formation[2,6,7,9]. The tumors are composed of two distinct cell types: a mononuclear cell population and in addition, osteoclastic tumor giant cells of uncertain lineage. The exceptional morphology and origin of the different cell types has been a matter of controversy since its first description in 1968. Recent evidence indicates that only the mononuclear cells constitute neoplastic tumor cells and that osteoclastic giant cells develop from secondary infiltrating cells.

Formation of osteoclast-like giant cells (OCGC) is speculated to result from fusion of bone-marrow derived mononuclear histiocytes/macrophages attracted to the tumor by chemotactic factors produced by the neoplastic cells[7,9,12,13]. Indeed, the CD-68/lysozyme reactivity of OCGCs suggests a histiocytic origin. Lack of mitoses and MIB-1 reactivity indicates a terminal stage of differentiation and non-neoplastic nature of the osteoclast-like giant cells, which are also K-ras negative[11]. Moderate staining of OCGCs with epithelial markers[20,25] is now explained by immunoreactivity of phagocytosed epithelial tumor cells[11,27]. The infiltrating mononuclear cells display

pleomorphism and neoplastic features and sometimes show features of epithelial tumors. However, other OGCTs lack epithelial differentiation[9,27]. Consequently, the term undifferentiated carcinoma with osteoclast-like giant cells has been proposed to more precisely describe these aggressive tumors.

Probably due to rapid tumor growth, osteoclast-like giant cell tumors of the pancreas only rarely present as small neoplasms. In more than 50 cases, in which tumor size was documented, only three neoplasias measured less than 3 cm. Typically OGCTs present as large tumors with cystic structures and necroses. The cystic component can become predominant, so that lesions may be misdiagnosed as pancreatic pseudocysts[3,4]. At time of diagnosis, more than 80% of tumors were already greater than 5 cm, 50% even greater than 10 cm. Most tumors arise in the head or body of the pancreas[1-15].

The differential diagnosis of pancreatic OGCTs includes cystic lesions like pancreatic cystadenomas, cystadenocarcinomas, serous and mucinous cystic tumors, pancreatic pseudocysts and also solid pancreatic tumors like ductal pancreatic carcinomas or neuroendocrine tumors. Solid tumors may be homogenous on computed tomographic imaging; however, they can also be very inhomogenous, as focal hemorrhage or necrosis is frequently found[9,12,15]. Vascularisation of these neoplasias has been described only in single cases. In one well-characterized patient, the tumor wall was slightly enhanced on contrast-enhanced CT[13]. Selective angiography demonstrated slight tumor staining[13].

In our patient with a pancreatic OGCT, contrast-enhanced sonography demonstrated a strong vascularisation within both the tumor and the liver metastases, making a pancreatic ductal carcinoma, which is typically only poorly vascularised, highly unlikely. A strong vascularised pancreatic neoplasm has to be differentiated from neuroendocrine tumors of the pancreas and from cystadenomas, which may also demonstrate strong vascularisation. In contrast, cystadenocarcinomas are generally poorly vascularised[28]. However, further studies are necessary to evaluate whether intense perfusion is a characteristic feature of OGCTs.

Osteoclast-like giant cell tumors of the liver are also generally large and inhomogenous neoplasias, ranging from 5 to 12 cm in size. Tumors typically feature necrotic or hemorrhagic regions and may also show cystic structures[16-27]. As published reports mainly focused on histopathology, radiological findings have only been documented in single cases. Magnetic resonance imaging in one case demonstrated a 10 cm large, fairly well circumscribed heterogenous solid mass with multiple fluid-like regions representing cystic components or necrosis on T1-weighted images[25]. Positron emission tomography scan in the same patient showed fluorine-18 fluorodeoxyglucose-uptake within the tumor[25]. In another patient, computed tomography described a 6 cm homogenous and hypervascularised tumor, which after resection presented as an inhomogenous tumor with hemorrhagic and necrotic areas[26].

In our patient, the liver tumor presented as an inhomogenous, cauliflower-like tumor with multiple small calcifications and a central stellar scar. However, contrast sonography revealed non-perfused tumor areas during the capillary and arterial perfusion phase, indicating necroses, thereby making a focal nodular hyperplasia highly unlikely[29,30]. Diagnosis of hemangioma or adenoma could be excluded by the spoke-like architecture of tumor arteries with centrifugal filling. The portal perfusion phase was characterized by a rapid decrease of perfusion within the tumor, indicating absence of portal vessels and thereby, together with the finding of a strong perfusion within the arterial phase, proving a malignant neoplasm[29,30].

A hepatocellular carcinoma was very unlikely, as the tumor demonstrated a very unusual vascular architecture and because there were no signs of cirrhosis. According to sonographic morphology and vascularisation pattern, the differential diagnosis included malignant primary liver tumors like peripheral cholangiocarcinoma (which, however, typically are poorly vascularized and do not show cysts or necroses), fibrolamellar carcinoma, epithelial hemangioendothelioma (which generally shows a multinodular structure) and a metastasis. However, a definitive sonographic diagnosis was impossible.

The average age of patients with osteoclast-like giant cell tumors of both pancreas and liver is around 60 years, ranging from 28 to 88 years. In pancreatic OGCTs, males and females appear to be affected in a fairly equal ratio[9,13]. The main symptoms are abdominal pain or discomfort and weight loss. Jaundice also frequently occurs if the tumor is located in the head. In cases of great cystic tumors, a palpable mass may be found. Invasion into adjacent structures is common. Nodal or intra-abdominal metastases are found in approximately 50% of patients at the time of diagnosis[1-15]. Overall, the prognosis of pancreatic OGCTs is unfavorable. As patients usually present with advanced diseases, complete resection can only rarely be performed. Leighton and Shiozawa reviewed 20 and 32 cases of pancreatic tumors, respectively, and determined the median survival rates to be less than 1 year. Interval to death or disease progression ranged from 4 mo to 5 years in these series[12,13]. In one patient, long-term survival of 15 years was documented; however, tumor recurrence within the pancreas after 10 years has also been reported[8]. Overall, the prognosis of pancreatic OGCTs is comparable to that of common ductal pancreatic carcinomas[12].

In contrast to pancreatic manifestations, OGCTs of the liver have been mostly described in male patients. Ten of 12 tumors (in one publication no data on patient's age and sex were reported)[27] occurred in men. The overall prognosis of OGCTs of the liver seems to be even worse than for pancreatic cases. Previous reports have demonstrated that these tumors are uniformly very aggressive and that survival ranges from 1 to 10 mo[16-26]. To date, there is only one report of chemotherapy and radiotherapy in the management of osteoclast-like giant cell tumors. Hood *et al* treated a patient with recurrent liver tumor with a combination of chemotherapy (5-fluorouracil and adriamycin), external beam radiation and radioimmunotherapy (I^{131}-labeled antiferritin immunoglobulin [IgG]), but could only achieve a partial response for several months[20]. In our case, the liver OGCT responded well to a combination

of carboplatin, etoposide and paclitaxel. With a combination of surgery, local ablative therapy and chemotherapy, our patient is currently the longest survivor reported. Due to epithelial features of the mononuclear neoplastic cells in some patients, agents like gemcitabine have been suggested as adjuvant or palliative therapy. As histology in our patients demonstrated undifferentiated tumors in both cases, we chose a polychemotherapy with cisplatin, etoposide and ifosfamide (PEI) and carboplatin, etoposide and paclitaxel, respectively. After three cycles of PEI, partial remission was observed in the patient with pancreatic OGCT. In the other patient with an OGCT of the liver, early tumor recurrence within the right lobe of the liver was observed 6 wk after surgical resection.

In conclusion, the histopathological features of these rare tumors have been precisely described in recent years, providing the basis for correct histological classification. OGCTs should also be included in the sonographic differential diagnosis of tumors of the pancreas and liver. B-mode sonography, as well as arterial perfusion of a liver OGCT resembles those of an FNH, reflecting its hepatic origin. However, demarcation of hemorrhagic necroses during the arterial and capillary phase together with a missing increase of signal intensity in comparison to the surrounding liver tissue during the portal and late phases allows exclusion of an FNH and diagnosis of a malignant neoplasm. For optimization of chemotherapy and other treatment strategies of osteoclast-like giant cell tumors, future studies should not only focus on histopathologic features, but also on diagnostic and therapeutic approaches.

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World J Gastroenterol 2006 December 28; 12(48): 7884
World Journal of Gastroenterology ISSN 1007-9327

ACKNOWLEDGMENTS

Acknowledgments to Reviewers of *World Journal of Gastroenterology*

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

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Meetings

MAJOR MEETINGS COMING UP

First Biennial Congress of the Asian-Pacific Hepato-Pancreato-Biliary Association
March, 2007
Fukuoka, Japan
http://www.congre.co.jp/1st-aphpba

American College of Gastroenterology Annual Scientific
20-25 October 2006
Las Vegas, NV

14th United European Gastroenterology Week, UEGW
21-25 October 2006
Berlin, Germany

APDW 2006: Asian Pacific Digestive Week 2006
26-29 November 2006
Lahug Cebu City, Philippines

EVENTS AND MEETINGS IN THE UPCOMING 6 MONTHS

Falk Symposium 151: Emerging Issues in Inflammatory Bowel Diseases
24-25 March 2006
Sydney - NSW
Falk Foundation e.V.
symposia@falkfoundation.de

10th International Congress of Obesity
3-8 September 2006
Sydney
Event Planners Australia
enquiries@ico2006.com
www.ico2006.com

Easl 2006 - the 41st annual
26-30 April 2006
Vienna, Austria
Kenes International

Prague hepatology 2006
14-16 September 2006
Prague
Foundation of the Czech Society of Hepatology
veronika.revicka@congressprague.cz
www.czech-hepatology.cz/phm2006

12th International Symposium on Viral Hepatitis and Liver Disease
1-5 July 2006
Paris
MCI France
isvhld2006@mci-group.com
www.isvhld2006.com

Falk Symposium 152: Intestinal Disease Part I, Endoscopy 2006 - Update and Live Demonstration
4-5 May 2006
Berlin
Falk Foundation e.V.
symposia@falkfoundation.de

Falk Symposium 153: Intestinal Disease Part II, Immunoregulation in Inflammatory Bowel Disease - Current Understanding and Innovation
6-7 May 2006
Berlin
Falk Foundation e.V.
symposia@falkfoundation.de

ILTS 12th Annual International Congress
3-6 May 2006
Milan
ILTS
www.ilts.org

Internal Medicine: Gastroenterology
22 July 2006-1 August 2006
Amsterdam
Continuing Education Inc
jbarnhart@continuingeducation.net

6th Annual Gastroenterology And Hepatology
15-18 March 2006
Rio Grande
Office of Continuing Medical Education
cmenet@jhmi.edu
www.hopkinscme.net

World Congress on Gastrointestinal Cancer
28 June 2006-1 July 2006
Barcelona, Spain
c.chase@imedex.com

International Conference on Surgical Infections, ICSI2006
6-8 September 2006
Stockholm
European Society of Clinical Microbiology and Infectious Diseases
icsi2006@stocon.se
www.icsi2006.se/9/23312.asp

7th World Congress of the International Hepato-Pancreato-Biliary Association
3-7 September 2006
Edinburgh
Edinburgh Convention Bureau
convention@edinburgh.org
www.edinburgh.org/conference

Society of American Gastrointestinal Endoscopic Surgeons
26-29 April 2006
Dallas - TX
www.sages.org

Digestive Disease Week 2006
20-25 May 2006
Los Angeles
www.ddw.org

Annual Postgraduate Course
25-26 May 2006
Los Angeles, CA
American Society of Gastrointestinal Endoscopy
www.asge.org/education

American Society of Colon and Rectal Surgeons
3-7 June 2006
Seatlle - Washington
www.fascrs.org

EVENTS AND MEETINGS IN 2006

10th World Congress of the International Society for Diseases of the Esophagus
22-25 February 2006
Adelaide
isde@sapmea.asn.au
www.isde.net

Falk Symposium 151: Emerging Issues in Inflammatory Bowel Diseases
24-25 March 2006
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Easl 2006 - the 41st annual
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Kenes International

VII Brazilian Digestive Disease Week
19-23 November 2006
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International Gastrointestinal Fellows Initiative
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24-27 February 2006
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Digestive Disease Week Administration
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www.cag-acg.org

Prague Hepatology 2006
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12th International Symposium on Viral Hepatitis and Liver Disease
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www.isvhld2006.com/

Falk Seminar: XI Gastroenterology Seminar Week
4-8 February 2006
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European Multidisciplinary Colorectal Cancer Congress 2006
12-14 February 2006
Berlin
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www.colorectal2006.org

Falk Symposium 152: Intestinal Disease Part I, Endoscopy 2006 - Update and Live Demonstration
4-5 May 2006
Berlin
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symposia@falkfoundation.de

Falk Symposium 153: Intestinal Disease Part II, Immunoregulation in Inflammatory Bowel Disease - Current Understanding and Innovation
6-7 May 2006
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25 February 2006-5 March 2006
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International Conference on Surgical Infections, ICSI2006
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World J Gastroenterol 2006 December 28; 12(48): 7886-7888
World Journal of Gastroenterology ISSN 1007-9327

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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 U S A* 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]

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]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303]

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]

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]

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9 Outreach: bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

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10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ Cell Tumour 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)

Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http//www.cdc.gov/ncidod/EID/eid.htm

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16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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