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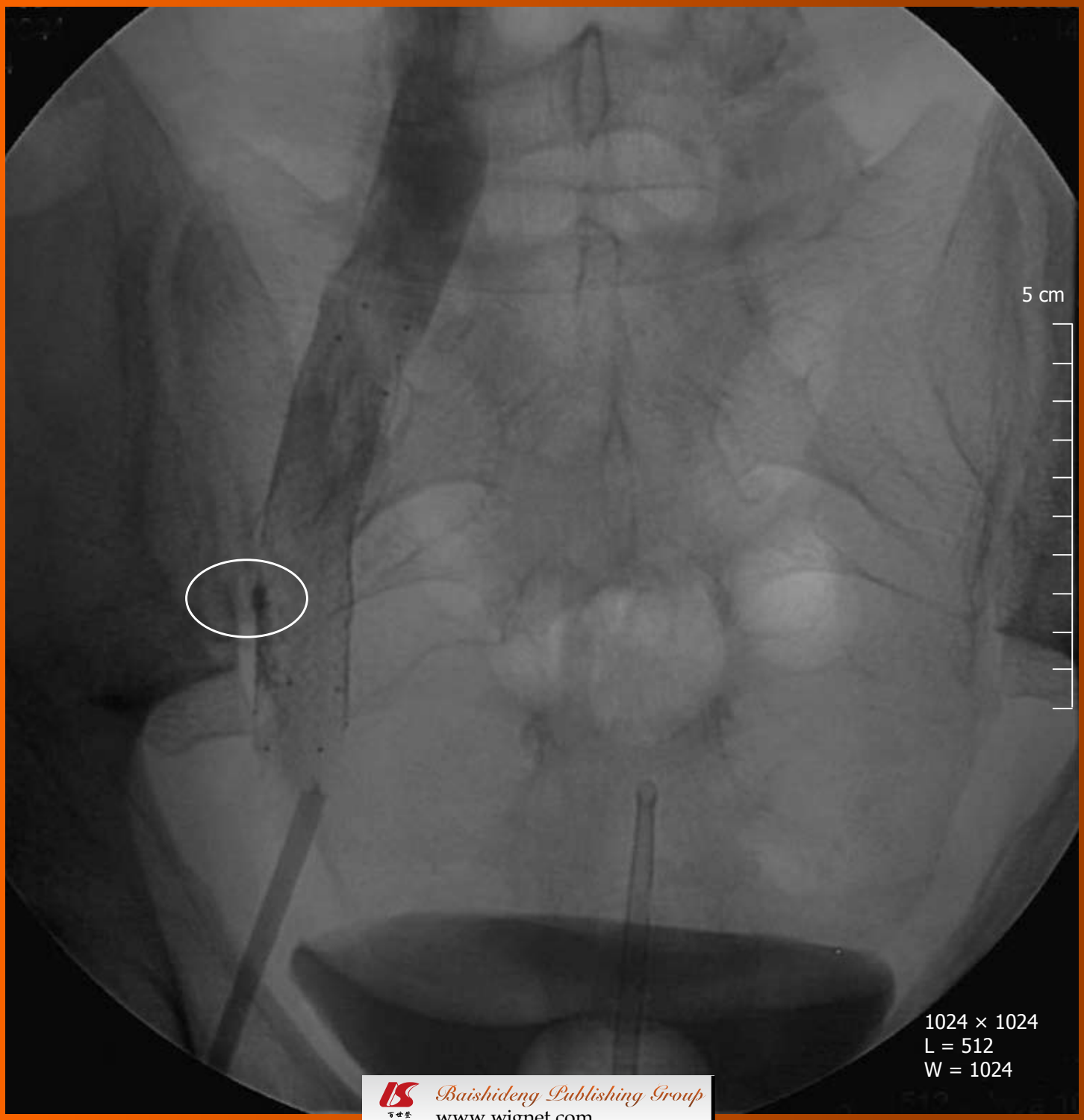

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EDITORIAL

Nonalcoholic fatty liver disease and the renin-angiotensin system: Implications for treatment

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Abstract

Nonalcoholic fatty liver disease (NAFLD) is the commonest liver disease in Western countries. Treatment of NAFLD is currently based on lifestyle measures and no effective pharmacologic treatment is available so far. Emerging evidence, mainly from animal studies, suggests that the renin-angiotensin-aldosterone system may be of major importance in the pathogenesis of NAFLD and indicates that angiotensin-converting enzyme inhibitors (ACE-I) and angiotensin receptor blockers (ARBs) as a potentially useful therapeutic approach. However, data from human studies are limited and contradictory. In addition, there are few randomized controlled trials (RCTs) on the effects of ACE-I or ARB in patients with NAFLD and most data are from retrospective studies, pilot prospective studies and post hoc analyses of clinical trials. Accordingly, more and larger RCTs are needed to directly assess the effectiveness of ACE-I and ARBs in NAFLD.

Key words: Nonalcoholic fatty liver disease; Non alcoholic steatohepatitis; Renin-angiotensin-aldosterone system; Angiotensin-converting enzyme inhibitors; Angiotensin receptor blockers; Fibrosis

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EDITORIAL

Nonalcoholic fatty liver disease (NAFLD) is the commonest liver disease in Western countries with an estimated prevalence in the general population of 20%-30%[1]. Treatment of NAFLD is currently based on lifestyle measures and no effective pharmacologic treatment is available so far. Emerging evidence, mainly from animal studies, suggests that the renin-angiotensin-aldosterone system (RAAS) may be of major importance in the pathogenesis of NAFLD and indicates that angiotensin-converting enzyme inhibitors (ACE-I) and angiotensin receptor blockers (ARBs) as a potentially useful therapeutic approach.

Within the circulating RAAS, the first step in the production of angiotensin Ⅱ (Ang Ⅱ) is the cleavage of angiotensinogen, produced in the liver, to Ang I by renin, which is released from the juxtaglomerular cells of the kidney. Then, the ACE converts Ang I into the physiologically active product of RAAS, Ang Ⅱ. Actions of Ang Ⅱ are mediated by the activation of the AT1 and the AT2 receptors (AT1R and AT2R). AT1R is in abundance in adult tissues whereas AT2 receptor is mainly expressed during fetal development and is upregulated in pathologic conditions. AT1R mediates all the major Ang Ⅱ-induced biological effects, such as the regulation of blood pressure, salt and water retention,

hormone secretion, renal function, as well as the autocrine and paracrine effects of Ang Ⅱ on cell proliferation and migration and extracellular matrix formation. AT2R is generally reported to mediate effects opposing and counterbalancing those mediated by AT1R *in vitro* as well as *in vivo*[2]. Ang Ⅱ is further metabolized by a variety of enzymes to the bioactive angiotensin fragments Ang Ⅲ (Ang2-8), Ang Ⅳ (Ang 3-8) and Ang (1-7). Ang Ⅲ (Ang 2-8) is formed by cleavage of Ang Ⅱ by aminopeptidase A and shares similar actions with Ang Ⅱ *via* AT1R and AT2R. Ang Ⅲ can be further metabolized by aminopeptidase M into Ang Ⅳ. Actions of Ang Ⅳ are mediated by AT4/(insulin-regulated aminopeptidase) receptor and include regulation of blood flow, inhibition of renal tubular sodium reabsorption, cardiac hypertrophy, angiogenesis and stimulation of endothelial cell expression of platelet activator inhibitor 1 (PAI-1)[3]. Angiotensin (1-7) is generated either from Ang I by endopeptidases or from Ang Ⅱ by ACE2. ACE2 also hydrolyzes Ang I to Ang-(1-9) which can be further metabolized to Ang-(1-7) by ACE. The effects of Ang-(1-7) are mainly mediated through the mas receptor and appear to counterbalance those of Ang Ⅱ. In particular, the ACE2-angiotensin-(1-7)-Mas axis appears to promote vasodilatation and to exert antiproliferative, anti-inflammatory, antifibrotic and antithrombotic actions[3,4].

Apart from the circulating RAAS, local RAAS have been identified in most organs and tissues, with diverse physiological effects exerted through autocrine and paracrine actions. These local RAAS have been implicated in multiple functions including cell growth, differentiation, proliferation and apoptosis, reactive oxygen species (ROS) generation, tissue inflammation, fibrogenesis and hormonal secretion[5]. The systemic and local RAAS are considered to interact and operate in a complementary and integrated way[6]. Experimental studies have demonstrated the presence of key elements of RAAS in normal liver and their up-regulation and redistribution in liver injury[7,8]. The AT1R, which is localized in hepatocytes, bile duct cells, hepatic stellate cells (HSCs), myofibroblasts, Kupffer cells and vascular endothelial cells, mediates most of the actions of Ang Ⅱ in the liver[4]. However, some studies also reported AT2R gene expression in liver tissue, suggesting that AT2R might have anti-fibrogenic effects in the liver[7,9].

Insulin resistance (IR) plays a central role in the pathophysiology of NAFLD and evidence from experimental studies underlines the crosstalk between RAAS and insulin signaling, resulting in the worsening of IR. Ang Ⅱ stimulates phosphorylation of serine residues in the insulin receptor beta-subunit and the p85 regulatory subunit of PI3-kinase, inhibiting the interactions between these components of the insulin signaling pathway[10]. Ang Ⅱ also induces generation of ROS mainly by activating the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and regulates the production of pro-inflammatory mediators, including tumor necrosis factor α, interleukin-6 (IL-6) and PAI-1, resulting in impairment of insulin signaling[11,12].

In vitro experiments and animal studies suggest that the effects of RAAS inhibition on glucose metabolism are due to vasodilation. In particular, ACE-I and ARBs-induced vasodilation increases the delivery of glucose and insulin to insulin-sensitive tissues and improves blood flow in pancreas, promoting insulin secretion. Preliminary data indicate enhanced insulin signaling, modulation of muscle fiber composition, decreased sympathetic activity and improved ionic balance as additional potential mechanisms implicated in the improvement of insulin sensitivity and secretion by RAAS-blocking agents. The beneficial effects of ARBs on IR could also be related to the selective stimulation of peroxisome proliferator-activated receptors (PPAR)-γ[13,14]. Furthermore, clinical trials have showed the ability of RAAS inhibition to prevent new-onset of diabetes mellitus. A recent meta-analysis including 100 848 patients showed a 20% reduction in the incidence of new onset diabetes with the use of ACE-Is or ARBs[15]. However, most studies included in this meta-analysis were post-hoc analyses and several were open label trials. In the Diabetes Reduction Assessment with Ramipril and Rosiglitazone Medication study, treatment with ramipril of patients with impaired fasting glucose levels or impaired glucose tolerance did not reduce the incidence of diabetes but increased regression to normoglycemia[16]. In the more recent Nateglinide and Valsartan in Impaired Glucose Tolerance Outcomes Research trial, treatment with valsartan of patients with impaired glucose tolerance was associated with a relative reduction of 14% in the incidence of diabetes[17].

Accumulating data suggest that the RAAS might play a role in the pathogenesis of hepatic fibrosis. Bataller demonstrated that activated HSCs express renin and ACE and synthesize Ang Ⅱ[7]. Ang Ⅱ activates HSC, promoting their differentiation into myofibroblasts and stimulates cellular proliferation contraction, through the release of intracellular calcium. Moreover, Ang Ⅱ up-regulates tissue inhibitor of metalloproteinases-1 (TIMP-1) mRNA expression and increases collagen and protein deposition in the extracellular matrix. The profibrogenic effect of Ang Ⅱ is also mediated *via* NADPH oxidase, which produces ROS. AT1R activation also increases the expression of vascular endothelial growth factor, promoting neoangiogenesis. Ang Ⅱ also exerts proinflammatory effects by up-regulating the synthesis of the pro-inflammatory cytokines IL-1 and IL-6 and the expression of the transcription factor nuclear factor kappa B. In addition, Ang Ⅱ stimulates the production of growth factors including transforming growth factor (TGF)-β1 and connective tissue growth factor. Moreover, Ang Ⅱ stimulates cell migration and concentration of activated HSCs at the site of hepatic injury[4,18,19].

Several studies in a variety of established animal models of hepatic fibrosis support the role of RAAS in liver fibrosis and the antifibrotic effects of RAAS inhibition. Treatment with ACE-I and ARBs in these models attenu-

ated steatosis and prevented the development of lobular inflammation and hepatic fibrosis. These effects appear to be due to the attenuation of oxidative stress and HSC activation and the down-regulation of pro-inflammatory and profibrotic cytokines[4,20]. Other potential mechanisms include suppression of growth factors and TIMP-1, increase in circulating adiponectin levels and reduction of macrophage infiltration[21-23].

Despite the supportive evidence of *in vitro* and *in vivo* studies, human data on the effects of RAAS inhibition on liver fibrosis are scarce. This can be attributed to the need for multiple liver biopsies and the slow progression of fibrosis necessitating studies with long-term follow-up. Regarding ARBs, a pilot study reported that prolonged administration of losartan (50 mg/d) for 18 mo was associated with downregulation of hepatic expression of fibrogenic genes in patients with hepatitis C[24]. Administration of losartan (50 mg/d) for 6 mo in patients with hepatitis C improved fibrosis stage compared with control patients[25]. Another study evaluated the efficacy of telmisartan (40 mg/d) and olmesartan (20 mg/d) in patients with NAFLD or chronic hepatitis C. Both drugs improved IR, measured by homeostasis model assessment of IR, and serum alanine aminotransferase levels but the benefit appeared to be greater with telmisartan[26]. In a controlled study, 30 patients with chronic hepatitis C were randomized to losartan (50 mg/d) and ursodeoxycholic acid (UDCA) or UDCA alone. Serum type Ⅳ collagen and plasma TGF-1 concentrations were significantly decreased in losartan group but there was no effect on fibrosis score[27].

Several studies compared both ACE-I and ARBs with other antihypertensive agents in patients with hepatitis C. In a retrospective study in liver-transplant recipients with hepatitis C recurrence, patients treated with ACE-I or ARBs showed reduced risk for cirrhosis and less liver fibrosis progression compared with patients who did not receive these agents[28]. In a more recent study in a similar population, administration of ARBs was associated with less progression of inflammation, but not fibrosis, whereas ACE-I had no effect on liver histology[29]. Another retrospective study showed that hypertensive patients with hepatitis C receiving ACE-I or ARBs had less fibrosis than hypertensive patients who received other antihypertensive agents[30]. In contrast to the previous studies, the Hepatitis C Antiviral Long-term Treatment against Cirrhosis Trial demonstrated no benefit of RAAS blockers in hepatic fibrosis[31]. In this study, patients with chronic hepatitis C and advanced hepatic fibrosis, who had failed to achieve a sustained virologic response after previous treatment, underwent serial liver biopsies at baseline, 1.5 years, and 3.5 years after randomization to maintenance therapy with peginterferon alfa-2a or to no treatment for 42 mo[31]. The trial showed no association between baseline use of RAAS inhibitors and liver fibrosis stage at baseline and use of ACE-I or ARBs did not slow progression of liver fibrosis during follow-up[31].

As far as patients with NAFLD or nonalcoholic steatohepatitis (NASH) are concerned, there are no studies that evaluated the effects of ACE-I in this population. Regarding ARBs, a preliminary study in 12 patients with NASH showed that losartan (50 mg/d) can improve biochemical parameters, liver steatosis and inflammation but had no effect on fibrosis[32]. In another pilot prospective study, the administration of losartan (50 mg/d) for 48 wk in 7 patients with NASH reduced circulating markers of hepatic fibrosis, plasma TGF-β1 levels, transaminase levels and improved hepatic necroinflammation and fibrosis[33]. In a larger study, 54 hypertensive patients with NASH were randomly assigned to either telmisartan (20 mg/d) or valsartan (80 mg/d). Both ARBs reduced transaminase levels and improved IR but this improvement was more profound in the telmisartan group, which also showed a significant decrease of NASH activity score and fibrosis. Valsartan did not improve liver histology except steatosis[34]. These differences on the effects on IR, transaminase levels and liver histology between ARBs could be attributed to the PPAR-γ-activating properties of telmisartan[35]. In addition, experimental studies demonstrated that telmisartan acts as a liver-specific partial PPAR-α agonist, has anti-inflammatory effects and modulates adipokine levels, by upregulating adiponectin levels and downregulating resistin levels[32,36]. Furthermore, structural differences between ARBs result in differences in their physicochemical properties and subsequently in their binding affinity to the Ang Ⅱ receptor[32]. On the other hand, a recent 12-mo randomized open-label study in 137 patients with NASH showed no additional benefit on liver histology with combination therapy of rosiglitazone and losartan (50 mg/d) compared with rosiglitazone alone[37].

Although there are no studies comparing ACE-I with ARBs in NAFLD, preliminary evidence indicates that treatment with ARBs result in greater improvement in insulin sensitivity and larger reduction in the risk for new onset diabetes mellitus. A meta-analysis showed that the number needed to treat to prevent one case of new onset diabetes is 100 and 50 with ACE-I and ARBs, respectively[15]. One possible explanation could be the inhibitory action of ACE-I on both AT1 and AT2 receptors, resulting in suppression of the counterbalancing effects of AT2 on the actions of AT1. Moreover, accumulating evidence suggests a beneficial role of ACE2/Ang-(1-7)/mas receptor axis since it appears to counterbalance the actions of Ang Ⅱ. Apart from ACE-I and ARBs which have been shown to up-regulate this pathway, new drugs that mimic the effect of Ang-(1-7) might represent a novel treatment of liver fibrosis[4].

In conclusion, the established role of both circulating and local RAAS on the pathogenesis of NAFLD and NASH created considerable interest on the effect of RAAS inhibitors since they are widely used, reasonably inexpensive, and with excellent safety profile. However, and despite the encouraging evidence from animal studies, data from human studies are limited and contradictory. In addition, there are few randomized controlled trials (RCTs)

on the effects of ACE-I or ARB in patients with NAFLD and most data are from retrospective studies, pilot prospective studies and post hoc analyses of clinical trials. Accordingly, more and larger RCTs are needed to directly assess the effectiveness of ACE-I and ARBs in NAFLD.

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EDITORIAL

Is nonalcoholic fatty liver disease the hepatic expression of the metabolic syndrome?

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Abstract

Nonalcoholic fatty liver disease (NAFLD) is generally considered as the hepatic manifestation of the metabolic syndrome (MS). Although there is no doubt that NAFLD is tightly linked to the MS, the diagnosis of NAFLD encompasses a broad range of histological entities and as a composite phenotype may be hindering attempts to understand the mechanistic basis of these variants. The awareness that NAFLD is not solely and invariably associated with the MS is a useful means to help direct future studies. We should be aware that mechanisms other than insulin resistance may contribute to the chronic inflammatory processes that underpin the development of liver fat accumulation and the subsequent architectural distortion of the liver. Further studies with special focus on hemoglobin as a risk factor for the development of NAFLD in the absence of MS should be performed.

Key words: Nonalcoholic fatty liver disease; Metabolic syndrome; Insulin resistance; Fibrosis; Hemoglobin

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INTRODUCTION

The epidemic of obesity is now a global and seemingly unstoppable phenomenon. Worldwide, the World Health Organization states that there are now over one billion overweight adults, of whom at least 300 million are obese[1]. In the wake of the obesity epidemic follow numerous comorbidities, including nonalcoholic fatty liver disease (NAFLD)[2]. NAFLD, which comprises a range of conditions from simple steatosis to nonalcoholic steatohepatitis (NASH) and cirrhosis, is the most common liver disease identified in Western countries[3]. The metabolic syndrome (MS), a potent risk factor for NAFLD, may represent a state in which the disturbances of metabolism that characterize fatty liver infiltration are already occurring prior to disease manifestation. However, debate rages as to whether the MS is, in fact, a useful concept in view of the lack of a unifying pathophysiology and issues of whether the sum of the components of the MS represent any greater risk than the components alone[4,5]. In addition, the fact that there is still no single internationally recognized definition of the MS reflects the diversity of opinion as to the purpose of defining the MS and whether there is a single principal underlying metabolic abnormality[6]. One widely held belief is that insulin resistance represents the unifying underlying pathological process resulting in both MS and NAFLD[7,8]. An alternative hypothesis is that the disturbances of lipid metabolism

seen in the MS result in a variety of secondary pathological processes which ultimately lead to fatty liver infiltration[9]. Therefore, an important issue in the assessment of the risk of NAFLD in patients with the MS is dependent on the criteria used to identify the MS itself[6]. However, there is another issue of utmost relevance that merits consideration. Although NAFLD is intricately intertwined with the MS, the definition of NAFLD includes only one component, i.e., liver fat content > 5%-10% by weight in the absence of excess alcohol consumption or any other liver disease[10]. Even more importantly, only 20% to 80% of patients with NAFLD fulfill the criteria for the MS[11]. Therefore, the presence of the MS alone does not sufficiently explain why some adults do have NAFLD. Starting from these premises, the identification of other factors that may explain this unexpected finding has key clinical implications.

NAFLD UNRELATED TO THE MS: HEMOGLOBIN AS THE KEY RISK FACTOR

To shed more light on the clinical and biochemical features of NAFLD unrelated with the MS, we have recently conducted a multicenter cross-sectional study in Turkey[12]. The purpose of the research was to determine if there were differences between patients with biopsy-proven NAFLD with and without a diagnosis of the MS. Our original hypothesis was that a detailed characterization of NAFLD patients without a diagnosis of the MS would be useful for identifying novel mechanisms of hepatic fat accumulation. Of a total of 357 consecutive patients with NAFLD recruited in the study, 214 met the ATP-Ⅲ criteria[13] for the MS while the remaining 143 did not. In NAFLD patients with the MS, insulin resistance and diabetes were independent predictors of NASH. Very intriguingly, the only variable independently associated with both NASH and severe moderate-to-severe fibrosis in NAFLD patients without the MS was hemoglobin. Receiver operating characteristic curve analysis also demonstrated that 144 g/L was the optimal hemoglobin cutoff value for a diagnosis of NASH in NAFLD patients without the MS, with a sensitivity and specificity of 75.5% and 71.3%, respectively[12]. If these results will be independently validated by future studies, we anticipate that liver biopsy should be recommended for patients with ultrasound-diagnosed NAFLD, no evidence of the MS, and hemoglobin levels higher than 144 g/L[12].

As expected, our data indicated that insulin resistance was significantly related to the presence of NASH and severe fibrosis in patients with biopsy-proven NAFLD; however, this association was chiefly confined in the subgroup of NAFLD patients with the MS. In contrast, hemoglobin levels were the most important independent predictor of both NASH and severe fibrosis in NAFLD patients without a diagnosis of MS. These findings are of interest and in keeping with a recent proteomic study which showed that free hemoglobin subunits positively associated with the severity of liver lesions in NAFLD[14].

In another large epidemiological study of 8985 Chinese subjects, Xu *et al*[15] reported that the prevalence rate of NAFLD increased with progressively higher hemoglobin concentrations. Notably, Yu *et al*[16] have also shown in an epidemiological study of 6944 apparently healthy subjects that increased baseline hemoglobin levels predict the incidence of NAFLD at a 3-year follow-up. Our study is the first to demonstrate that hemoglobin is the main independent predictor of the severity of the liver lesions in patients with biopsy-proven NAFLD without MS[12]. However, the exact mechanisms underlying this association remain to be determined. Previous studies have demonstrated that increased hemoglobin concentrations lead to increased blood viscosity, thereby raising peripheral resistance and reducing blood flow and perfusion[17,18]. In turn, a reduced blood perfusion to the liver has been suggested to accelerate fibrosis[19]. Furthermore, increased iron itself can increase liver damage by oxidative stress and lipid peroxidation[20]. The possible mechanisms leading to increased hemoglobin levels in NASH and in NAFLD subjects with advanced fibrosis need additional study, but it might be a consequence of hepatic hypoxia resulting in a stimulation of erythropoietin production.

CONCLUSIONS AND PERSPECTIVES

Is NAFLD just the mirror of the MS at the hepatic level? Clinical studies have clearly shown that the answer to this key question is "no". Although there is no doubt that NAFLD is tightly linked to the MS, the diagnosis of NAFLD encompasses a broad range of histological entities and as a composite phenotype may be hindering attempts to understand the mechanistic basis of these variants. The awareness that NAFLD is not solely and invariably associated with the MS is a useful means to help direct future studies. We should be aware that mechanisms other than insulin resistance may contribute to the chronic inflammatory processes that underpin the development of liver fat accumulation and the subsequent architectural distortion of the liver.

Inferring clinically relevant insights from the complex picture of the quantitative changes in expression levels of circulating molecules remains a major challenge in NAFLD. Ideally, NAFLD biomarkers should be accessible in a minimally invasive way through assaying the serum, plasma, or blood. Initial exploratory studies aimed at the discovery of biomarkers are frequently performed using high-throughput proteomics-based platforms[21]. Currently, hemoglobin is clearly the most widely replicated proteomic biomarker of NAFLD. Accordingly, it has been identified as a biomarker of NAFLD in two independent proteomic studies[14,16] and then validated using distinct analytical methods in large and independent replication cohorts[12,15,16]. In our previous study[12], we have also shown that high hemoglobin levels were associated not only with the presence of NASH but also with the extent of hepatic fibrosis. Decreased blood flow to the liver due to increased hemoglobin levels may indeed in-

duce hepatic hypoxia and a profibrotic response[22].

Further studies with special focus on hemoglobin as a risk factor for the development of NAFLD in the absence of MS should be performed. Additional research will also be required to determine whether or not treatment strategies chiefly focused on reducing hemoglobin may contribute to the modulation of systemic inflammatory response or the development of common metabolic diseases. An important objective of such studies will be also to assess the diagnostic accuracy of hemoglobin for predicting NASH and liver fibrosis while properly adjusting for confounding effects from clinical risk factors and drug exposures.

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REVIEW

Management of alcoholic hepatitis: Current concepts

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Abstract

Alcoholic hepatitis is a devastating form of acute liver injury seen in chronic alcohol abusers with significant morbidity and mortality. It is a multisystem disease that is precipitated by ingesting large quantities of alcohol with genetic and environmental factors playing a role. Prognostic criteria have been developed to predict disease severity and these criteria can serve as indicators to initiate medical therapy. Primary therapy remains abstinence and supportive care, as continued alcohol abuse is the most important risk factor for disease progression. The cornerstone of supportive care remains aggressive nutritional support, and although acute alcoholic hepatitis has been extensively studied, few specific medical therapies have been successful. Corticosteroids remain the most effective medical therapy available in improving short term survival in a select group of patients with alcoholic hepatitis; however, the long-term outcome of drug therapies is still not entirely clear and further clinical investigation is necessary. While liver transplantation for acute alcoholic hepatitis have demonstrated promising results, this practice remains controversial and has not been advocated universally, with most transplant centers requiring a prolonged period of abstinence before considering transplantation. Extracorporeal liver support devices, although still experimental, have been developed as a form of liver support to give additional time for liver regeneration. These have the potential for a significant therapeutic option in the future for this unfortunately dreadful disease.

Key words: Alcholic hepatitis; Acute liver injury; Abstinence; Nutrition; Corticosteroids; Transplantation; Extracorporeal liver support

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INTRODUCTION

Alcoholic hepatitis is a multi-system disease seen in patients who have abused large quantities of alcohol over an extended period of time, often years. The development of alcoholic hepatitis is complex and dependent on a variety of genetic and environmental factors. In general, men who ingest more than 100 g of ethanol daily for more than 5 years are at highest risk of developing alcoholic hepatitis; however, women may develop alcoholic hepatitis after ingesting smaller amounts of ethanol for shorter periods of time. Alcoholic hepatitis can adversely affect multiple organ systems: the gastrointestinal system, central nervous system, hematologic system, cardiovascular system, and renal system. Symptoms

are non-specific and may include fatigue, right upper quadrant abdominal pain, anorexia, weight loss, jaundice, and fever. There is usually a history of recent binge drinking. Clinical signs may include tender hepatomegaly often with a systolic hepatic bruit, jaundice, fever, ascites, and encephalopathy in more severe cases. Physical stigmata of underlying chronic liver disease may be present including spider angiomata, splenomegaly, palmar erythema, gynecomastia, parotid gland enlargement, testicular atrophy, and Dupuytren's contractures. Laboratory tests classically show modest elevations in aspartate aminotransferase (AST) and alanine aminotransferase (ALT) (typically not greater than 300 mg/dL), with AST greater than ALT, bilirubin levels as high as 30 mg/dL, marked coagulopathy, leukocytosis, anemia, and renal failure in the most severe cases. Diagnosis can be made on clinical and biochemical grounds and liver biopsy is not routinely needed to confirm the diagnosis. Based on the American Association for the Study of Liver Disease (AASLD) guidelines set forth in 2010 for alcoholic liver disease, it is recommended that for patients with a clinical diagnosis of severe alcoholic hepatitis for whom medical treatment is contemplated, or for those in whom reasonable uncertainty exists regarding the underlying diagnosis, a liver biopsy should be considered[1].

Patients with severe alcoholic hepatitis frequently deteriorate within a few days to weeks in the hospital with progressive hepatic and renal failure despite supportive measures. Severe alcoholic hepatitis is associated with a high short-term mortality rate, approaching that of fulminant hepatic failure without orthotopic liver transplantation. Indeed, nearly one-half of patients with severe alcoholic hepatitis die within one month of hospitalization. In an effort to determine prognostic criteria for these gravely ill patients with acute alcoholic hepatitis, Maddrey *et al*[2] found significant independent associations with prothrombin time prolongation, peak serum bilirubin and mortality. Using these parameters, the discriminant function (DF) was devised as a disease specific prognostic scoring system [DF = 4.6 × prothrombin time (seconds prolonged more than control) + serum total bilirubin (mg/dL)]. Patients with the most severe disease, as defined by a DF greater than 32 have the highest risk of dying, with a one month mortality of 30%-50%[3]. Furthermore, spontaneous hepatic encephalopathy or the model for end-stage liver disease score greater than 18 in patients with acute alcoholic hepatitis carries a similar poor prognosis and has been used along with the DF to predict disease severity. Moreover, in patients who survive the initial hospitalization, alcoholic hepatitis is a well-known precursor of alcoholic cirrhosis, especially in patients who continue to drink.

TREATMENT

Abstinence

The primary and most effective intervention for alcoholic hepatitis is complete abstinence from alcohol consumption. Abstinence is the single most important factor in the prevention of disease progression and can improve survival. However, survival decreases with concurrent portal hypertension and cirrhosis. Furthermore, abstinence is crucial for those patients with advanced liver disease who may eventually require orthotopic liver transplantation. Several medications have been investigated in hopes of maintaining alcohol abstinence. Disulfiram was one of the first United States Food and Drug Administration approved agents; however, its use has largely been abandoned due to poor tolerability and lack of supporting data[4]. Alternatively, short term treatment with the opiod antagonist naltrexone has been shown to reduce the risk of alcohol relapse[5]. Similarly, acamprosate, an inhibitory neurotransmitter similar to gamma-aminobutyric acid, has been shown to decrease the rate of alcohol relapse and maintain abstinence[6]. Nonetheless, some patients with alcoholic hepatitis will progress despite abstinence and supportive medical care. Thus, several potential therapies have been studied in an attempt to achieve a more favorable disease course.

Supportive care

In addition to abstinence, the most vital therapy for a patient with acute alcoholic hepatitis is supportive care. This includes intensive care unit monitoring and intubation for airway protection when appropriate, especially in the setting of advanced hepatic encephalopathy. Alcohol withdrawal must be treated with benzodiazepines as needed. Intravenous fluids should be administered with electrolyte, mineral and vitamin supplementation, including replacement of phosphate, potassium, magnesium, multivitamins, thiamine and folate. A single dose of vitamin K may be given, but will not correct the coagulopathy if due to underlying hepatic synthetic dysfunction. Administration of fresh frozen plasma should be reserved only for active hemorrhage.

Hepatotoxic medications should be avoided, as should nephrotoxic medications, including aminoglycosides, angiotensin converting enzyme inhibitors and non-steroidal anti-inflammatory medications. Sepsis is a common cause of mortality in these gravely ill patients; however, it is often difficult to determine the presence of concomitant infection, as fever and leukocytosis are often encountered in acute alcoholic hepatitis, even in the absence of infection. Accordingly, a vigilant search for infection with appropriate cultures, including diagnostic paracentesis is crucial.

Nutrition

Patients with severe protein-calorie malnutrition have been found to have a significantly higher mortality compared to those who are only mildly malnourished[7]. In fact, hepatic dysfunction in alcoholics was initially thought to be due to nutritional deficiencies. Thus, supplemental nutrition with high-calorie, high-protein diets administered either enterally or parenterally to these malnourished patients was advocated at the outset in hopes to improve outcomes. However, subsequent clinical tri-

als of supplemental nutrition administered to patients with acute alcoholic hepatitis have not been shown to improve mortality in this disease. Despite these findings, high-calorie, high-protein formulations have been shown to improve nutritional parameters during the acute illness and high catabolic state[8-10]. Therefore, it may be worthwhile to utilize supplemental enteral nutrition during the acute phase of illness, especially in anorexic patients who do not meet their calculated daily requirements[11]. Newer and more expensive branched-chain amino acid formulations have been proposed to have a lower incidence of hepatic encephalopathy. However, even standard amino acid preparations have not been shown to cause hepatic encephalopathy in cirrhotic patients with portal hypertension[9,12]. Thus, branched-chain amino acid formulations do not likely offer an efficacy advantage and given the large discrepancy in price, are not cost-effective. It is also important to replace deficiencies in certain vitamins and minerals, including vitamins A, D, pyridoxine, thiamine, folate and zinc.

Corticosteroids

In view of the dismal prognosis associated with alcoholic hepatitis, many drugs have been investigated as potential mediators to alter the clinical course of this disease. Due to their well-known anti-inflammatory effects, corticosteroids have been the most extensively studied medication in patients with alcoholic hepatitis, many randomized controlled trials have produced inconsistent results[2,13-23]. Thus, in 1990, Imperiale *et al*[24] reviewed 11 of the earlier trials conducted between 1971 and 1989 in hopes of reaching a consensus statement on the efficacy of corticosteroids for the treatment of alcoholic hepatitis[2,13-22]. In this landmark meta-analysis, steroids were determined to be most beneficial in a subset of patients with severe acute alcoholic hepatitis and spontaneous hepatic encephalopathy, reducing the risk of short-term mortality to 0.66 for a protective efficacy of 34%. In contrast, Christensen *et al*[25] determined that glucocorticoids had no statistically significant beneficial or harmful effect. This meta-analysis found a high probability of publication bias in this area and cautioned against the routine use of glucocorticoids in patients with acute alcoholic hepatitis. The most recent meta-analysis on this subject confirmed that while corticosteroids were not beneficial for all patients with alcoholic hepatitis, there was a survival benefit in patients with severe disease, defined as the presence of spontaneous hepatic encephalopathy and/or DF $\geqslant$ 32[26]. In a reanalysis of individual data from the last three randomized placebo controlled trials of corticosteroids, Mathurin *et al*[27] found a significant increase in one month survival for patients with severe alcoholic hepatitis (DF $\geqslant$ 32) treated with steroids (85% *vs* 65%). Thus, five patients needed to be treated to prevent one death. In an attempt to predict those individuals not responding to corticosteroids, a recently developed model was created using six clinical variables to calculate a Lille score (www.lillemodel.com). After 7-d of corticosteroids, a Lille score > 0.45 indicates a poor response to therapy and a 6-mo mortality of > 75%[28].

In accord with the recommendations by the American College of Gastroenterology and AASLD, corticosteroids use cannot be supported in patients with alcoholic hepatitis with concomitant gastrointestinal hemorrhage, pancreatitis, active infection or renal failure, as these patients were excluded in many of the clinical trials advocating corticosteroid treatment[1,29]. Prednisolone 40 mg per day orally for four weeks followed by a taper or discontinuation is favored over prednisone, which requires hepatic conversion to the active prednisolone.

Of note, corticosteroids have not been shown to improve long-term survival, as follow-up in clinical studies have rarely extended beyond a few months, due to the high initial mortality rate associated with this disease. In a study comparing prednisolone to enteral feedings for 28 d for severe alcoholic hepatitis, there was no significant difference between groups in the treatment phase (25% *vs* 31%, respectively)[30]. However, 37% of the survivors in the steroid group died during the one-year follow-up compared with 8% of the survivors in the enteral feeding group. Most of these deaths were due to infections. Thus, despite the many years of investigation, the long-term benefits of corticosteroids in severe alcoholic hepatitis remain unclear.

Tumor necrosis factor alpha inhibition

Pentoxifylline is an oral phosphodiesterase inhibitor which also decreases tumor necrosis factor alpha production, which is known to be elevated in patients with alcoholic hepatitis. In a randomized controlled trial with 101 patients with severe alcoholic hepatitis defined as a DF $\geqslant$ 32, pentoxifylline was given to 49 patients at a dose of 400 mg three times daily for 4 wk[31]. The remaining 52 patients received placebo. As expected, the control group had a mortality rate of 46%, but the treatment group demonstrated a significant improvement in survival with a mortality rate of 25%. Moreover, the reduction in mortality appeared to correlate with a significantly lower incidence of hepatorenal syndrome in the pentoxifylline group compared to the control group (8% compared with 35%). The findings of this study are encouraging and need to be confirmed with long-term follow-up.

Other clinical trials investigating anti- tumor necrosis factor agents including infliximab and etanercept given in conjunction with corticosteroids for acute alcoholic hepatitis showed no mortality benefit[32,33].

Phosphatidylcholine

In an effort to prevent alcohol-induced hepatocyte mitochondrial dysfunction, supplementation with the phospholipid phosphatidylcholine has been studied. In alcohol-fed baboons, phosphatidylcholine prevented the progression of pericentral and interstitial fibrosis to septal fibrosis and cirrhosis[34]. Currently, a large randomized controlled trial is under way in humans.

S-adenosysl-methionine

S-adenosyl-methionine (SAMe) helps to maintain mitochondrial glutathione stores in alcoholic liver disease. At a dosage of 1200 mg per day for 2 years, SAMe exhibited a decrease in mortality and a delay in transplantation exclusively in Child-Turcotte-Pugh class A and B cirrhotics[35]. It has not been studied in acute alcoholic hepatitis.

Antioxidants

Vitamin E may have a beneficial antioxidant role in alcoholic liver disease, but the outcomes of trials have been disappointing[36,37]. Milk thistle, which contains the antioxidant silymarin may provide a benefit, albeit small, in Child-Turcotte-Pugh class A alcoholic cirrhotics who continue to drink alcohol[38]. Most recently, the antioxidant effect of N-acetylcysteine was used in conjunction with glucocorticoids in patients with severe alcoholic hepatitis and demonstrated an increased 1 mo survival, although 6 mo survival was not improved[39].

Propylthiouracil

Alcohol induces a hypermetabolic state with pericentral hypoxia, similar to that seen in hyperthyroidism. Propylthiouracil has been tried in the hopes of reversing this hypermetabolic response and curtailing hepatocellular damage. In a randomized controlled trial with 67 patients with severe alcoholic hepatitis, propylthiouracil at 300 mg per day for 6 wk had no benefit on morbidity or mortality[40]. Subsequently, in a long-term randomized trial with 310 patients, propylthiouracil demonstrated a significant mortality benefit, especially in patients with the most severe alcoholic hepatitis (55% compared with 25% placebo). However, propylthiouracil only conferred a benefit to those who remained abstinent[41]. Furthermore, concerns regarding propylthiouracil-induced hypothryroidism have diminished the fervor for the use of propylthiouracil in alcoholic hepatitis.

Anabolic steroids

Androgens have been studied in an effort to improve the general nutritional status of patients with alcoholic hepatitis. In a large, multicenter population of United States veterans, oxandrolone at a dose of 80 mg per day for 1 mo did not affect short-term survival, but did increase survival at 6 mo in a subgroup of patients with moderate, but not severe alcoholic hepatitis[21]. Unfortunately, these results have been confirmed in subsequent studies. Hence, the use of oxandrolone for acute alcoholic hepatitis cannot be routinely recommended.

Colchicine

Colchicine has been examined in patients with cirrhosis due to its effects on collagen and hepatic fibrogenesis. Moreover, it inhibits leukocyte migration and function and has positive effects on cytokine production related to fibroblast proliferation. Consequently, a randomized controlled trial was conducted in a population of 72 hospitalized patients with severe alcoholic hepatitis[42]. At the standard dose of 1 mg orally per day for 1 mo, colchicine had no beneficial effect on morbidity, mortality or biochemical tests of liver function.

Amlodipine

Calcium channel blockers have been shown to have a hepatoprotective effect in animal models of alcohol-induced liver injury. However, a randomized, double-blind, placebo-controlled trial showed no conclusive evidence that amlodipine benefits patients with acute alcoholic hepatitis[43].

Insulin and glucagon

Insulin and glucagon have been known to enhance hepatic regeneration after partial hepatectomy in experimental animals. An early randomized clinical trial involving 50 patients administered insulin and glucagon infusions for the treatment of acute alcoholic hepatitis showed promise[44]. However, subsequent larger studies have failed to show a clinical benefit, including short-term or long-term survival benefit[45,46]. In fact, significant hypoglycemia became problematic, which limited the utility of this intervention in acute alcoholic hepatitis.

Transplantation

In the setting of acute alcoholic hepatitis, orthotopic liver transplantation is highly controversial. Yet, patients receiving liver transplantation for this disease have generally resulted in good outcomes. In fact, it has been suggested that transplantation even for acute alcoholic hepatitis is successful with comparable outcomes to that of patients transplanted for decompensated alcoholic cirrhosis alone[47,48]. Most recently, Mathurin *et al*[49] demonstrated an improved 6 mo survival in patients with a first episode of severe alcoholic hepatitis not responding to medical therapy who underwent early liver transplantation compared to those who did not (77% *vs* 23%). Conversely, other investigators demonstrated a poor prognosis after transplantation with rapidly progressive liver injury with active alcoholic liver disease[50]. Combining the high risk of recidivism in patients with acute alcoholic hepatitis along with the national shortage of donor organs, orthotopic liver transplantation has not been advocated for acute alcoholic hepatitis.

Initially, there was hesitation to transplant patients for decompensated alcoholic cirrhosis due to the perception that the disease was self-inflicted and concerns about post-transplantation compliance[51]. Nevertheless, it is now clear that in appropriately selected patients with decompensated alcoholic cirrhosis, orthotopic liver transplantation provides an excellent prognosis, with outcomes similar to other nonalcoholic chronic liver diseases. In these appropriately screened patients, recidivism after transplantation still occurs in one-fifth to one-half of patients, but only 5%-7% return to excessive drinking[52,53]. The most predictive variable of recidivism has been proposed to be a period of abstinence before transplantation[54]. A documented 6 mo of pre-transplantation

abstinence is usually required as a minimal criterion for liver transplantation listing[55]. Other factors including social support and functional level are also extremely important to maintain abstinence. Current endeavors are underway to incorporate multiple variables to better risk stratify patients and standardize selection criteria for orthotopic liver transplantation in this population of patients. It is conceivable similar criteria could be used to determine appropriate liver transplant candidacy in the setting of acute alcoholic hepatitis as well. Notwithstanding, it has been estimated that only 5% of patients with end stage liver disease related to alcohol are formally evaluated to be considered for liver transplantation[56].

Extracorporeal liver support

Although they are still experimental, extracorporeal liver support devices have been developed as a form of liver support to give additional time for liver regeneration. One approach utilizes membranes and adsorbents that can remove toxins associated with liver failure, similar to hemodialysis in patients with end-stage renal disease. This type of extracorporeal liver support with molecular adsorbents recirculating system (MARS) uses a dialysis module in which the patient's blood is dialyzed across an albumin-impregnated membrane, where detoxification can occur. Substances larger than 50 kDa, such as growth factors and essential hormones, are not removed. This device has shown promise in patients with acute liver failure superimposed upon chronic liver disease[57]. Recently, the MARS treatment was performed on 8 patients with severe acute alcoholic hepatitis superimposed on biopsy-proven cirrhosis[58]. These patients received 3 to 12 courses of 6 h of MARS treatment and encouraging results were seen in liver biochemistry, renal function, encephalopathy and mortality. However, the lack of power in this study does not allow definite conclusions. Nonetheless, a multi-center, randomized clinical trial is under way to study the efficacy of MARS treatment in acute alcoholic hepatitis.

CONCLUSION

Alcoholic hepatitis is a severe form of acute liver injury associated with significant morbidity and mortality. Although, prognostic criteria have been developed to help predict disease severity, it remains a challenge to treat. However, the primary intervention remains abstinence, as this is the most important risk factor for disease progression and is required if transplantation is eventually needed. Vigilant supportive care and adequate nutrition is crucial to help overcome this devastating illness. Several medications have been studied in an effort to alter the clinical course of this disease. Corticosteroids have been effective in reducing the short-term mortality in a subset of patients with severe alcoholic hepatitis with hepatic encephalopathy. Pentoxifylline also confers a significant short-term benefit for severe alcoholic hepatitis. Of note, despite more than 30 years of randomized controlled trials, no specific pharmacologic agent has clearly demonstrated a long-term survival benefit. Though outcomes in patients receiving liver transplantation for alcoholic hepatitis are similar to those transplanted for nonalcoholic liver diseases, liver transplantation in this patient population remains highly controversial. Extracorporeal liver support devices are still in their developmental infancy and may be an option in the future.

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REVIEW

Hepatitis C virus-related hepatocellular carcinoma: An insight into molecular mechanisms and therapeutic strategies

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Abstract

Hepatitis C virus (HCV) infects more than 170 million people worldwide, and thereby becomes a series global health challenge. Chronic infection with HCV is considered one of the major causes of end-stage liver disease including cirrhosis and hepatocellular carcinoma. Although the multiple functions of the HCV proteins and their impacts on the modulation of the intracellular signaling transduction processes, the drive of carcinogenesis during the infection with HCV, is thought to result from the interactions of viral proteins with host cell proteins. Thus, the induction of mutator phenotype, in liver, by the expression of HCV proteins provides a key mechanism for the development of HCV-associated hepatocellular carcinoma (HCC). HCC is considered one of the most common malignancies worldwide with increasing incidence during the past decades. In many countries, the trend of HCC is attributed to several liver diseases including HCV infection. However, the development of HCC is very complicated and results mainly from the imbalance between tumor suppressor genes and oncogenes, as well as from the alteration of cellular factors leading to a genomic instability. Besides the poor prognosis of HCC patients, this type of tumor is quite resistance to the available therapies. Thus, understanding the molecular mechanisms, which are implicated in the development of HCC during the course of HCV infection, may help to design a general therapeutic protocol for the treatment and/or the prevention of this malignancy. This review summarizes the current knowledge of the molecular mechanisms, which are involved in the development of HCV-associated HCC and the possible therapeutic strategies.

Key words: Hepatitis C virus; Hepatocellular carcinoma; Cirrhosis; Fibrosis; Inflammation; Carcinogenesis

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INTRODUCTION

Chronic infection with hepatitis C virus (HCV) is considered one of the major causes of end-stage liver disease including cirrhosis and hepatocellular carcinoma. HCV

infects more than 170 million people worldwide[1], and thereby becomes a series global health challenge. In the last decades, understanding the molecular pathogenesis of HCV infection was hampered by the lack of a suitable infection model, however, the establishment of both HCV replicons[2,3] and small animal models[3], helped to a better understanding the molecular mechanisms of both life cycle and the etiopathogenesis of the virus.

HCV is an enveloped virus with positive-sense RNA genome of 9.6 kb that encodes for a single polyprotein[4]. This single polyprotein can be cleaved by both viral and cellular proteases into 10 mature proteins including, structural (Core, E1, E2/p7) and nonstructural (NS2, NS3, NS4A, NS4B, NS5A and NS5B) proteins (Figure 1).

The natural course of HCV infection is the progression to fibrosis and cirrhosis, and subsequently to hepatocellular carcinoma (HCC) in a significant proportion of HCV infected patients[5-7]. Thus, beside its role in the cause of chronic infection that is mostly associated with the development of fibrosis and cirrhosis, HCV may play an integral role in the development of HCC *via* mechanisms mediated by viral proteins-host cell interaction[8,9].

Because of the close association of cirrhosis with HCV-related HCC, the molecular mechanisms of HCV-mediated carcinogenesis are intensively discussed in the context of liver diseases, such as chronic inflammation, steatosis and fibrosis. However, these liver diseases seem to be the main cause for the development of cirrhosis[10-12]. Although the multiple functions of the HCV proteins and their impacts on the modulation of the intracellular signaling transduction processes, the drive of carcinogenesis during HCV infection, is thought to result from the interactions of viral proteins with host cell proteins[13-16]. Thus, the induction of mutator phenotype, in liver, by the expression of HCV proteins provides a key mechanism for the development of HCV-associated HCC.

MOLECULAR MECHANISMS OF ONCOGENIC PROTEINS OF HCV

Based on their proliferative potential that is widely documented *in vitro* and *in vivo*, some of HCV viral proteins including core, NS3, NS5A and NS5B have been shown to possess an oncogenic potential[17-19]. These documented oncogenic potenial results mainly from the interference of HCV viral proteins with cellular proteins, which are responsible for the regulation of cell cycle control[20]. Under normal physiological conditions, the cell cycle progression is regulated by consecutive activation of cyclin and cyclin-dependent kinase (CDK) complexes[21]. For example, active cyclin-CDK complexes in G1 results in the phosphorylation of the retinoblastoma family of proteins (pRb, p130 and p107), leading the activation of the members of transcription factor family E2F, and subsequently the upregulation of cellular genes, which are characteristic for the progression of G1 phase of the cell cycle[22]. In addition to cyclin-CDK complexes, the regulation of these checkpoints are p53 and rb pathways-dependent activation[23,24].

Up on the transcriptional activation of the cycline-dependent kinase inhibitor p21, during G1/S transition, by p53, p21 becomes available to bind and to inhibit CDK2, leading to cell cycle arrest[21]. Thus, anti-growth signals, such as checkpoint activation can play an essential role by limiting the replication of oncogenic viruses, in response to viral infection including HCV.

The interference of cellular proteins and HCV core protein is considered a major risk factor for the progression of HCC. As widely reported, the expression of HCV core protein in a transgenic mouse model was found to be sufficient to induce tumor formation in liver[25]. In addition, HCV core can trigger the activation of peroxisome proliferator-activated receptor alpha that, in turn, may contribute to HCC[26-28]. Also the expression of HCV core protein was found to promote the immortalization of primary human hepatocytes as well as to reverse replicative senescence[29]. In addition to the activation of telomerase in the immortalized hepatocytes, the HCV core protein was found to increase the expression of interleukin (IL)-6, gp130, leptin receptor, and signal transducer and activator of transcription 3[29]. However, the upregulation of these genes, in response to the expression of HCV core protein, thought to be involved in the regulation of c-myc and cyclin D1, and subsequently leading to the promotion of cellular transformation[30].

The role of NS3 in the neoplastic transformation of hepatocytes *in vivo* and *in vitro*[17,31-33]. Also, the enhancement of transformation and tumorigenicity upon transfection with HCV NS3 DNA has been reported in the non-tumorigenic mouse fibroblast cell line NIH 3T3 into nude mice[34]. Moreover, the HCV NS3 C-terminal-deleted protein showed both transforming and oncogenic potential[35]. The expression of the NS3 protein in human hepatocytes was found to induce transformed characters with reduced population doubling time as well as anchorage-independent growth and tumor development that is associated with the increased phosphorylation of extracellular regulated protein kinases and p38 proteins[36]. Also, the NS3 protein has been shown to form complexes with p53[19], and to inhibit p21 promoter activity[37].

The oncogenic potential of HCV NS5A protein has been shown to be mediated by suppression of the cell cycle regulatory gene p21 in response to its interaction to p53[38-40]. In addition, NS5A protein has been reported to suppress the expression of the mitotic spindle protein ASPM through the PKR-p38 signaling pathway, as well as the induction of aberrant mitoses, chromosome instability and HCC[41].

In addition to its ability to form a cytoplasmic complex with Rb in infected cells[42], the HCV NS5B-dependent downregulation of Rb results in the enhancement of E2F-dependent transcription as well as the promotion cellular proliferation[21]. Also, the cell-cycle checkpoint, the mitotic spindle checkpoint, is a target for HCV NS5B. Since the interaction of the HCV polymerase NS5B with Rb results in the degradation of Rb and activates the MAD2 promot-

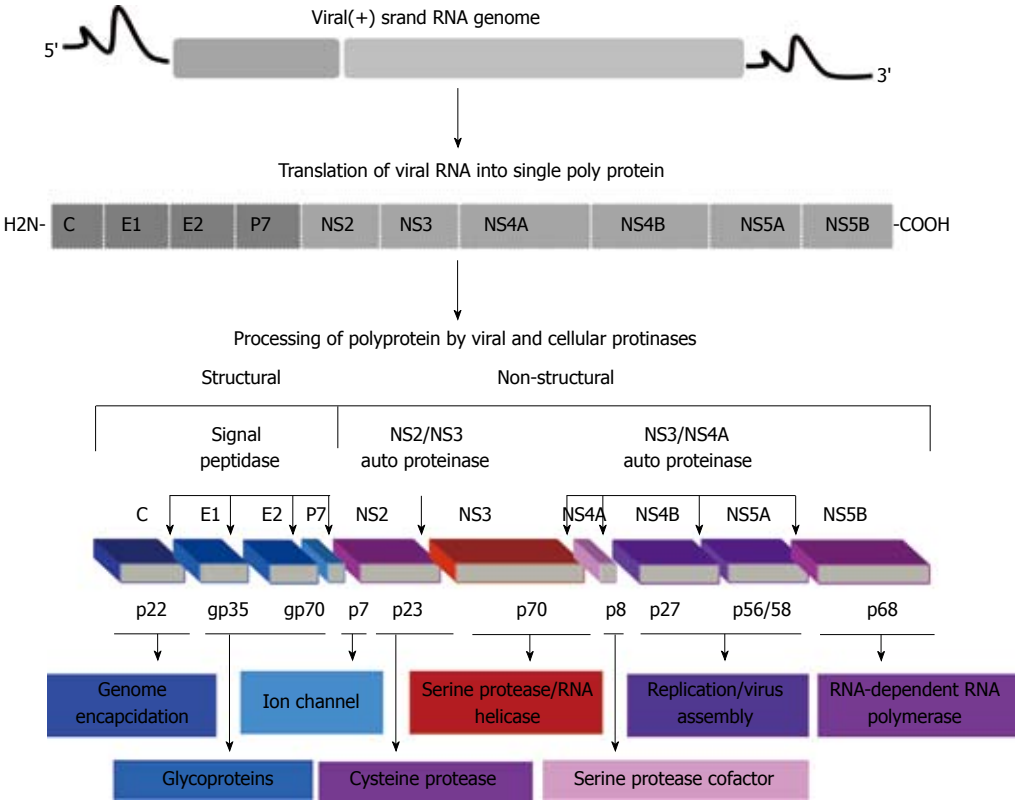

Figure 1 Hepatitis C virus genome including 5' and 3' noncoding regions, and the long open reading frame encoding for polyprotein precursor of 3010 amino acids. This polyprotein precursor can be cleaved functionally by co- and post-translationally processes mediated by cellular and viral proteases into ten different products, including structural and non-structural proteins. The structural proteins core (C), envelop 1 (E1) and E2 are located in the N-terminal third, whereas, the non-structural/replicative proteins (NS2, NS3, NS4A, NS4B, NS5A, NS5B) are located in the remainder of the polyprotein. Putative functions of the cleavage products are shown.

er[43]. Thus, the loss of host-cell genomic stability due to deregulation of Rb pathway may result from viral infection.

MOLECULAR MECHANISM OF HCV-ASSOCIATED CHRONIC INFLAMMATION

Chronic inflammation, which generally associated with the increased proliferation of tissue cells, and an increased rate of random mutations, leads mostly to chromosomal instability[44-48], and ultimately to both tumor progression and invasion[8,49,50]. Also, the correlation between chronic inflammation and the promotion of carcinogenesis has been reported in several clinical studies dealing with HCV-associated liver cirrhosis and HCC[51-53]. Accordingly, the interaction of viral proteins with cellular factors in host cells[21,54], and the augmentation of chronic liver disease during the course of HCV infection, suggests a central role for viral proteins in the regulation of chronic inflammation leading to the initiation and subsequently progression of HCC[55-59]. Thus, HCV-associated chronic inflammation seems to result from the dysregulation of cell cycle control[60] and the loss of tumor-suppressor gene functions[61], together with the induction of the proinflammatory cytokines, such as tumor necrosis factor (TNF)-α. Since the significant increase of proinflammatory cytokines was noted in HCV-expressing cells[31,62], and in clinical samples including liver biopsies and sera of HCV-infected patients[54,63]. Therefore, the association between chronic inflammation and the development of HCC, during the infection with HCV is considered. Indeed, the chronic inflammatory process of HCV infection that is thought to be responsible for the promotion of the increased mutation rate in the regenerating hepatocytes, and thereby contributes to the development of HCC[45-48]. In contrast, the rare occurrence of HCC in auto-immune hepatitis[64,65], indicates that the inflammation alone cannot be the reason for a high incidence of HCC in HCV-infected patients[66-68]. Although the role of HCV-associated chronic inflammation is considered to be the primary inducer of liver fibrosis and cancer, the molecular mechanisms whereby the chronic inflammation mediates the progression of liver fibrosis and subsequently HCC are not fully understood. As widely reported, chemokines produced in the liver during HCV infection are involved mainly in the regulation of migration of activated T cells from the periphery to infected parenchyma[69]. More important, these chemokines and their receptors are associated not only with viral control, but also with immune-mediated liver inflammation[70]. Accordingly, in a hepatotropic viral infection in humans, a marked intrahepatic non-specific mononuclear infiltrate during viral persistence was reported[71], suggesting an essential role for the intrahepatic chemoattraction of non-specific T cells in the modulation of liver damage[69]. Thus, besides their functional role in viral clearance,

chemokines and their receptors are implicated in the development of chronic tissue inflammation. In fact, the modulation of these pathways seems to be essential for generating an efficient immune response, as well as for the regulation of the inflammatory process during the course of the chronic infection with HCV, a viral strategy to escape from immune control[72].

Generally, chemokines and their receptors are the main actor in the regulation of multistep pathway of inflammatory processes, which are responsible for the migration of lymphocytes to the liver[73,74]. In chronic hepatitis C, the expression of different chemokines in the liver has been documented in several studies[75-78]. The most reported chemokines include CXCL10 that is produced by hepatocytes and sinusoidal endothelial cells[76,77], CXCL9 and CXCL11, which are increased in the serum and liver of patients with chronic hepatitis C[76,79]; CCL5 that is elevated in chronic hepatitis C and it is produced by hepatocytes, sinusoidal endothelial cells and biliary epithelium[80]. However, the expression of all these chemokines in the liver can be induced directly by HCV. Since the induction of CXCL10, CXCL9 and CCL5, in hepatocytes, by HCV proteins, including NS5A and core has been reported[81]. Although the dominant role of chemokines in the modulation of HCV-associated inflammation, the precise mechanisms, which are involved in the regulation of HCV-associated chronic inflammation still remain to be discussed in detail. The mechanisms that thought to be involved in the regulation of HCV-associated chronic inflammation are induction of IL-1β, IL-6 and TNF-α by HCV core and NS3 proteins, indirect induction of CXCL10 and CXCL9 by HCV core and NS5A proteins.

MOLECULAR MECHANISMS OF HCV-ASSOCIATED FIBROSIS

Generally, a variety of adverse stimuli including viruses such as HCV can trigger fibrogenesis. However, the ability of HCV and its proteins to induce fibrosis is mediated either direct by the interference of HCV proteins with various cellular pathways[82-84], or indirect *via* steatosis[26,85], or type 2 diabetes[86-88] - dependent mechanisms, which finally lead to the deregulation of released cytokines[89-91].

As widely recognized, the excess synthesis and deposition of extracellular matrix (ECM) that is mainly directed by the induction of cytokine release, is mostly associated with the increased severity of liver disease[92,93]. As a result, the matrix metalloproteinase (MMPs) including, MMP-1, -2, -3, -8, -9, -12, -13 and -14, become inactive and fail to remove excess ECM[94-96], and subsequently disturb the balance between fibrogenesis and fibrolysis in the liver[97-100], an evidence for the development of liver fibrosis. Therefore, the inhibition of MMPs in response to repeated liver injury can lead to the dysfunction of ECM, and subsequently to undesired tissue remodeling, architectural disruption and a fibrogenic response.

As known, the source of fibrogenic cytokines and growth factors in liver is activated liver macrophages, such as Kupffer cells, proliferating bile ductular epithelia, endothelia, mononuclear cells, and myofibroblasts[101-103]. Therefore, the stimulation of hepatic stellate cells and provascular fibroblasts by fibrogenic cytokines and growth factors mediates their transformation into myofibroblasts, the main source of collagens, MMPs and tissue inhibitor of MMPs, resulting in the accumulation of ECM that is responsible for the balance between fibrogenesis and fibrolysis in the liver[104-106]. However, a proposed model for the development of liver fibrosis during the course of HCV infection is outlined in Figure 2.

Transforming growth factor β1 (TGF-β1) is the most prominent profibrogenic cytokine that can be released from any cell type during inflammation, tissue regeneration and fibrogenesis[107-111]. Thus, besides its multiple functions, the TGF-β1 is strongly involved in the regulation of the production and deposition of the major ECM proteins[112,113].

As known, fibrosis results from the deposition of ECM material around the liver parenchyma. This deposition is mediated by the promotion of liver fibrogenesis that results mainly from either the inflammation of liver stellate cells or from hepatocyte damage in response to the generated reactive oxygen species (ROS) by Kupffer cells[114,115].

Besides the role of epithelial-mesenchymal transition paradigm in the development of fibrosis and HCC, epithelial cells are considered important mediators for progressive fibrosis and HCC[116]. During the progression of chronic liver diseases, such as HCV infection, hepatocytes undergo transition from tumor-suppressive pSmad3C pathway, a characteristic pathway of epithelial cells, to JNK/pSmad3L pathway that is, in turn, mediates the activation of myofibroblasts leading to the promotion of liver fibrosis and subsequently increasing the risk of cancer[117-119]. The loss of both epithelial homeostasis and acquisition of migratory mesenchymal phenotype are known to be essential for tumor invasion[120-122]. In this context, the role of HCV-induced JNK pathway[17,36,54], is thought to be essential for the regulation of Smad3L-depndent signaling[36,123]. Thus, the possible interaction of Smad3L-depndent signaling with oncogenic pathways including, the activator protein 1 may be responsible for the augmentation of the mesenchymal phenotype of hepatocytes[124]. Thus, the interaction of HCV core with Smad3[125,126], and the subsequent inhibition of TGF-β-induced Smad3 transcriptional activity provides evidence for the contribution of HCV core protein in the regulation of TGF-β signaling and its downstream biological responses seem to be a possible mechanism for the development of HCV-associated HCC[54,127].

The elevation of TGF-β2 production in the sera of HCV-infected patients or in core-expressing liver cells[54], in HCC biopsies[128], besides the significant cell proliferation in HCV core-expressing cells[36,54,123], suggest a central role for TGF-β signaling pathway in the regulation of HCV-associated HCC. Although the functional

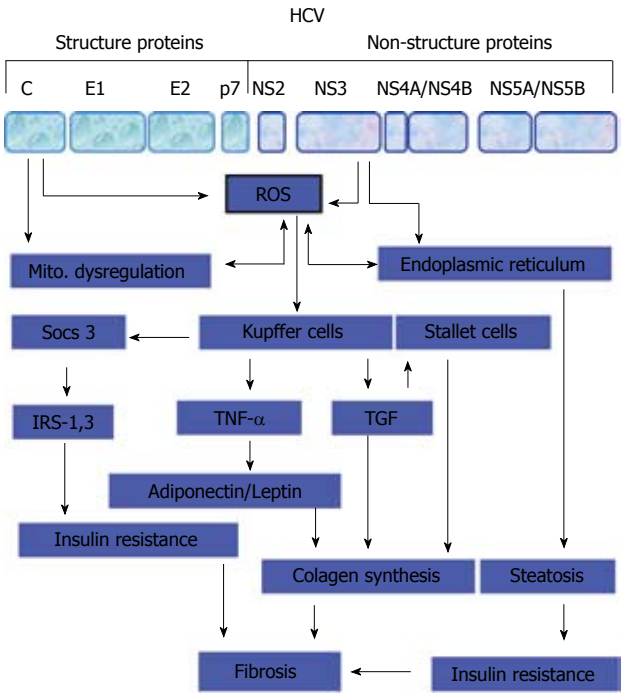

Figure 2 Pathogenesis of liver fibrosis in chronic hepatitis C infected patients. Potential mechanisms that thought be involved in the regulation of hepatitis C virus -associated hepatic fibrosis. HCV: Hepatitis C virus; ROS: Reactive oxygen species; IRS: Insulin receptor substrate; TNF: Tumor necrosis factor; TGF: Transforming growth factor.

role of TGF-β in liver tumorigenesis as well as the implication of EMT in HCC development is not well elucidated, the contribution of HCV oncogenic potential in the course of hepatocarcinoma is widely documented[17,36,54,129]. However, the possible factors and mediators, which are thought to be involved in the regulation of HCV-associated fibrosis are ROS, TGF-β1, TNFα, epidermal growth factor (EGF), insulin-like growth factor, micro integral membrane protein (TiMP)-1, TiMP-3, MMP-1, MMP3, MMP-8.

MOLECULAR MECHANISMS OF HCV-ASSOCIATED HCC

HCC is the only malignancy whose occurrence in patients is associated with the appearance of risk factors, such as chronic liver inflammation and cirrhosis[130-132]. However, the extensive epidemiological studies performed in the last decades led to the identification of major risk factors of HCC and thereby helped to understand the pathogenesis of HCC[133-135]. Although the advances that made in the understanding of HCC pathogenesis, little is known about the molecular mechanisms of this malignancy. The most changes that occur in liver tissues are thought to result from either viral infection or the exposure to hepatotoxic agents leading to significant changes in the cellular signaling pathways and their target genes that are responsible in the regulation of tumor formation. These pathways include Wnt/β-catenin[8,136], p53[137,138], pRb[139-141], mitogen-activated protein (MAP) kinases[142,143], stress signaling[144-147], Ras[148-150], epidermal growth factor receptor[151-153], TGF-β[54,154], and JAK/STAT[155].

Wnt/β-catenin pathway has been reported to be involved in the regulation of HCC development in response to viral infection including HCV[156]. Also, the up-regulation of frizzled-7 and dephosphorylation of β-catenin is frequently observed in HCC[157-160]. Therefore, the targeted inactivation of Wnt pathway is considered a potential therapeutic target for the prevention or the ablation of HCV-associated HCV. Moreover, the increase of the mutation in β-catenin in HCC patients in response to either HCV infection[161] or the exposure to aflatoxin[162,163], provides evidence for the involvement of Wnt pathway in the regulation of HCV-associated HCC.

The tumor suppressor *P53* gene, which can be inactivated by single point mutation[164,165], is one of the most studied proteins in the context of tumor development. Although the expression of this protein at normal levels in most tumors, under normal physiological conditions, the level of cellular p53 is low. The alteration of the expression level of p53 in response to either intracellular or extracellular stress signals can lead to significant changes that mostly vary from down regulation to up-regulation[164-166]. However, the loss of p53 function as tumor suppressor protein is controlled by defects in p53 signaling.

Retinoblastoma, pRb1 is a major cellular barrier to cancer development that controls cell cycle progression through a mechanism including, the repression of the E2F transcription factor family of proteins[167,168]. The phosphorylation of pRb and subsequently G1/S cell cycle transition is mainly correlated with activation of CDKs in different tumor types including, HCC[20,169-173]. In according, HCV core protein-induced acceleration of liver cells was found to be associated with activation of CDKs, inhibition of pRb and the up regulation of E2F1[20,54]. In addition, there is strong correlation between the loss of pRB and the inhibition of functional p53 in HCV core expressing cells[21], as well as in different tumor types including HCC[169-171]. The inhibition of CDK inhibitors p16INK4A, p21(WAF1/CIP1), and p27Kip1 in response to frequent mutation, or HCV infection was found to be associated with carcinogenesis of most HCC cases[172,173]. Also, the disruption of pRb pathway in various tumor types including, HCC has been reported in several studies[20,174], an evidence for the critical role of pRB in carcinogenesis.

Although HCV is a single-stranded RNA virus, and its genome is never integrated into the genome of hepatocytes[175], and no known oncogenic properties have been reported for its genes, a significant portion of HCV-infected patients with induced cirrhosis has been shown to develop HCC[20]. Thus, HCV-induced oncogenesis seems to result from the interference of HCV proteins with the intracellular signal transduction processes *via* mechanism includes dysregulation of cell cycle control.

Core protein, the most viral protein that is widely reported to interact with several intracellular signal transduction pathways, and thereby orchestrate their function,

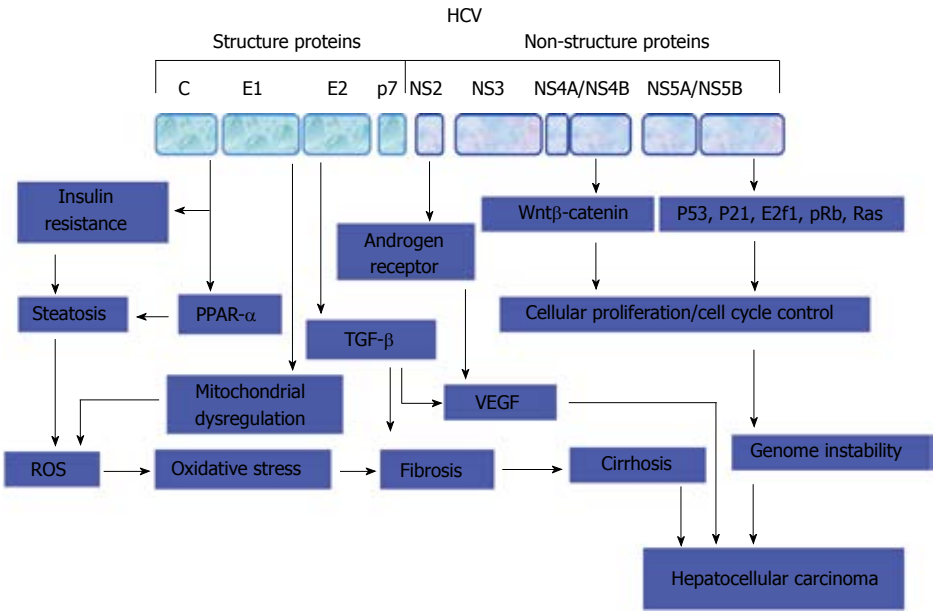

Figure 3 Molecular mechanisms of hepatitis C virus-mediated hepatocarcinogenesis. Key steps that thought to be involved in the development of hepatitis C virus-associated hepatocellular carcinoma. HCV: Hepatitis C virus; ROS: Reactive oxygen species; IRS: Insulin receptor substrate; TGF: Transforming growth factor; VEGF: Vascular endothelial growth factor; PPAR: Peroxisome proliferator-activated receptor.

as oncogenic mediator, by indirect activation of TNF-α receptor[176,177], Raf-1 kinase[173,178], MAP kinase[36,179], E2F1/Rb[21] and nuclear factor kappa B[180,181] pathways. Also, the inhibition of TNF-α-induced apoptosis and the modulation of other cytokines activities during the course of HCV infection may prolong survival of infected hepatocytes, and subsequently leads to the accumulation of genetic damages that mediate the processes of the malignancy[182-184].

Although the direct involvement of HCV core protein in the development of HCC has been demonstrated in transgenic mice[25,185], the mechanism(s) whereby HCV core triggers HCC is not completely addressed in human. Apart from HCV core protein, the role of other viral proteins such as, nonstructural proteins NS5A and NS3 in the development of hepatocarcinogenesis is less clear[17-19,38-41]. A suggested model for the development of HCV-associated HCC is outlined in Figure 3. Also, the possible mechanisms of HCV-associated HCC are: (1) activation of cellular oncogenes such as, Ras, c-Myc, E2F1 by HCV proteins; (2) inactivation of tumor suppressor genes such as p21, p53, Rb by HCV proteins; and (3) HCV proteins-induced dysregulation of Wnt/β-catenin, MAPK, JAK/STAT, PI3K/Akt, EGF-β, TGF-β pathways.

THERAPEUTIC STRATEGIES OF HCV-ASSOCIATED HCC

Currently, the available HCC therapy is limited and usually with no clinical benefit for patients with advanced disease[186-188]. Although surgery or liver transplantation can successfully cure small or slow-growing tumors, the success is hampered because of donor organ shortage as well as the rapid and frequent recurrence of HCC in the transplanted liver[189-191].

Despite the potentially curative and palliative approaches are available for the treatment of HCC[192,193], there is no effective systemic chemotherapy for HCC treatment. Apart from limited benefit of the available therapies, the choice of the HCC treatment depends on several factors including, cancer stage, resources, and practitioner expertise.

Several anticancer agents including, sorafenib have shown promise in the treatment of patients with HCC[194]. Sorafenib is a small molecule multikinase inhibitor with antiproliferative, antiangiogenic and pro-apoptotic properties. Although its limited benefit for patients with advanced HCC and compensated cirrhosis, the treatment with sorafenib is associated with the increase in overall survival[195,196]. However, the relative success with sorafenib, despite the commonly reported side effects, has prompted its clinical utilization as a relevant therapeutic either alone or in combination with other treatments[197,198]. In addition, the reliability of sorafenib as relevant therapeutic approaches for HCC encouraged to test other small molecules, such as brivanib and erlotinib[199-202], and monoclonal antibodies, such as bevacizumab,and cetuximab[203,204] for their therapeutic potential in patients with hepatocellular carcinoma. Based on the successful clinical development of sorafenib in HCC treatment, the era of the molecularly targeted agents undergoes active clinical development.

Although the efficacy of antiviral therapy on HCV viral status and underlying liver function in patients is still unclear, antiviral treatment may render patients with HCV-related HCC to tolerate HCC treatments and thereby may improve prognosis[205,206]. However, based on its success, the clinical management of chronic HCV can improve the prevention of the late recurrence of HCC. Whereas, the high viral load has been shown to be an HCC recurrence risk that can be common to all HCV carriers-independent from their HCcAg status, alanine aminotransferase (ALT) levels, and stage of chronic infection[207,208]. Interferon-α therapy was found to reduce significantly the risk for hepatocellular carcinoma, especially among virologic or

biochemical responders[209]. For example, patients with sustained biochemical response, independent from viral load, were at reduced risk for HCC, when compared with patients with sustained virologic response[210,211]. However, the degree of these reduced HCC risk is thought to be variable related to ALT levels[212,213], suggesting that the reduced risk of HCC recurrence is not associated only with disappearance of viremia, but also with amelioration of hepatic inflammation.

The key signal transduction pathways, which are involved in the regulation of the pathogenesis of HCV-associated HCC are considered a roadmap for the development of clinical relevant approach for the treatment or the prevention of HCV-associated HCC. Currently, the targeted therapies, which are developed for the pathways that are mentioned in the context of HCV-mediated HCC development are either in clinical development or already proved for clinical use. These include therapies that target endothelial growth factor receptor, insulin growth factor 1[214,215], vascular endothelial growth factor receptor 1-3[216], in addition to those target c-MET[217], Ras/Raf and MEK[169,173], Akt/mTOR[218], pathways. Other signaling pathways such as, Jak-STAT, and TGF-β[54], need more attention to investigate their clinical relevance and therapeutic potential in the treatment of HCC or HCV-associated HCC.

CONCLUSION

Chronic infection with HCV can lead to cirrhosis and hepatocellular carcinoma. Although the allegation of clinicians and researchers that the presence of cirrhosis is the main output for the development of HCC in individuals with chronic HCV infection, the direct role of HCV infection in the development of HCC in non-cirrhotic individuals has been suggested.

Generally, the induction of cancer is a multistep-dependent mechanism. In HCV infection, however, some of these steps might be bypassed during the development of HCV-associated HCC. Therefore, the overall effects that can be achieved by the expression of viral proteins including core protein, even in the absence of a complete set of genetic aberrations, are essential for carcinogenesis. Apart from conventional process of the induction of HCC, a plausible explanation might be given for many unusual events that take place in HCV-infected patients. The incidence of HCC in patients with HCV is known to correlate with the progression of liver fibrosis. However, the degree of liver fibrosis and the status of the infection may influence the risk of HCC occurrence in HCV-infected patients. Although HCC without cirrhosis in HCV-infected patients is rare, the direct implication of viral proteins in the development of HCC has been recently reported. Thus, the contribution of the viral infection to the development of HCC is thought to result from chronic hepatitis and/or cirrhosis.

Although there is no evidence that HCV by itself is oncogenic, the development of HCC in non-cirrhotic HCV-infected individuals is less frequent, so that a direct oncogenic effect of viral proteins is considered.

Commonly, in patients with HCV-related HCC, the tumors seem to be solitary, smaller sized, and encapsulated, when compared to those of hepatitis B virus (HBV)-related HCC. Because of the most of HCC that occurs in patients with chronic hepatitis and liver cirrhosis, is associated with infection with HBV or HCV, the treatment of these hepatitis viruses with anti-viral agents and chemoprevention approaches may decrease the risk of HCC.

However, the key signal transduction pathways, which are involved in the regulation of the pathogenesis of HCV-associated HCC, are considered a roadmap for the development of clinical relevant approach for the treatment or the prevention of HCV-associated HCC.

Thus, the development of novel targeted therapies based on the inhibition of the signaling pathways, which are directly involved in the regulation HCV-mediated initiation, progression and invasion of HCC may provide a better picture of the clinical utility and treatment options for patients with HCV-associated HCC.

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ORIGINAL ARTICLE

Role of cytokine receptor-like factor 1 in hepatic stellate cells and fibrosis

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Abstract

AIM: To elucidate the role of cytokine receptor-like factor 1 (CRLF1) in hepatic stellate cells and liver fibrosis.

METHODS: Rat hepatic stellate cells (HSCs) were isolated by Nykodenz gradient centrifugation and activated by culturing *in vitro*. Differentially expressed genes in quiescent and culture activated HSCs were identified using microarrays. Injections of carbon tetrachloride (CCl_4) for 4 wk were employed to induce liver fibrosis. The degree of fibrosis was assessed by Sirius red staining. Adenovirus expressing CRLF1 was injected through tail vein into mice to achieve overexpression of CRLF1 in the liver. The same adenovirus was used to overexpress CRLF1 in quiescent HSCs cultured *in vitro*. Expression of CRLF1, CLCF1 and ciliary neurotrophic factor receptor (CNTFR) in hepatic stellate cells and fibrotic livers was analyzed by semi-quantitative reverse transcription-polymerase chain reaction and Western blotting. Expression of profibrotic cytokines and collagens was analyzed by the same method.

RESULTS: CRLF1 is a secreted cytokine with unknown function. Human mutations suggested a role in development of autonomous nervous system and a role of CRLF1 in immune response was implied by its similarity to interleukin (IL)-6. Here we show that expression of CRLF1 was undetectable in quiescent HSCs and was highly upregulated in activated HSCs. Likewise, expression of CRLF1 was very low in normal livers, but was highly upregulated in fibrotic livers, where its expression correlated with the degree of fibrosis. A cofactor of CLRF1, cardiotrophin-like cytokine factor 1 (CLCF1), and the receptor which binds CRLF1/CLCF1 dimer, the CNTFR, were expressed to similar levels in quiescent and activated HSCs and in normal and fibrotic livers, indicating a constitutive expression. Overexpression of CLRF1 alone in the normal liver did not stimulate expression of profibrotic cytokines, suggesting that the factor itself is not pro-inflammatory. Ectopic expression in quiescent HSCs, however, retarded their activation into myofibroblasts and specifically decreased expression of type Ⅲ collagen. Inhibition of type Ⅲ collagen expression by CRLF1 was also seen in the whole liver. Our results suggest that CLRF1 is the only component of the CRLF1/CLCF1/CNTFR signaling system that is inducible by a profibrotic stimulus and that activation of this system by CLRF1 may regulate expression of type Ⅲ collagen in fibrosis.

CONCLUSION: By regulating activation of HSCs and expression of type Ⅲ collagen, CRLF1 may have an ability to change the composition of extracellular matrix in fibrosis.

Key words: Hepatic stellate cells; Liver fibrosis; Cytokine receptor-like factor 1; Cardiotrophin-like cytokine factor 1; Ciliary neurotrophic factor receptor; Type Ⅲ collagen

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INTRODUCTION

Cytokine receptor-like factor 1 (CRLF1) is a secreted protein belonging to the family of cytokine type I receptors and has homology to two other secreted receptors: IL12B and EBI3[1,2]. Both of these receptors are implicated in regulating immune response by T-lymphocytes. Expression of CRLF1 is induced by tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6) and γ-interferon and it is expressed in lymphatic tissue, placenta, stomach and lungs[3]. CRLF1 forms a heterodimer with cardiotrophin-like cytokine factor 1 (CLCF1, also known as B-cell stimulating factor 3) and this complex binds to the ciliary neurotrophic factor receptor (CNTFR)[4]. CNTFR is a non-signaling component of the heterotrimeric receptor composed of CNTFR, interleukin 6 signal transducer (IL6ST) and LIFR[5]. The IL6ST and LIFR subunits of this heterotrimeric receptor are also components of IL-6 receptor. However, while IL-6 receptor binds IL-6, CNTFR/IL6ST/LIFR receptor binds either CLRF1/CLCF1 dimer or ciliary neurotrophic factor (CNTF)[6]. Like IL-6 receptor, the CNTFR/IL6ST/LIFR receptor signals to activate STAT/ERK pathway[7]. CNTF is required for development of central nervous system and has neurotrophic activity for motor neurons. Knock out of *CNTF* gene results in progressive muscular atrophy and loss of motor neurons[8]. Elson *et al*[4] have shown that CLRF1/CLCF1 dimer is a competitive inhibitor for binding of CNTF to CNTFR/IL6ST/LIFR receptor.

The physiological role of CRLF1 is unknown. Mutations in *CRLF1* gene were associated with cold sweat syndrome type 1 and Crisponi syndrome[9-14]. Cold sweat syndrome type 1 is characterized by profuse sweating induced by cold and craniofacial deformities[12,14]. Crisponi syndrome is associated with dismorphic facial features, facial muscle contractions, scoliosis and hyperthermia[9,13]. Interestingly, mutation of CLCF1 causes cold sweat syndrome type 2, which is similar to cold sweating syndrome type 1[15], suggesting the common signaling defect of the CLRF1/CLCF1 pathway. These syndromes implicated the role of CLRF1/CLCF1 in the function of the autonomic nervous system. Mice lacking the *CLRF1* gene were unable to suckle and died from starvation few days after birth[16]. No craniofacial deformations were observed and the reason for suckling defect is unknown.

CLRF1 was found to be expressed at high levels in osteoarthritic human knee cartilage and was upregulated by stimulating mouse chondrocytes by transforming growth factor-β (TGF-β)[17]. CRLF1/CLCF1 complex promoted the proliferation of chondrocyte precursors and suppressed the expression level of aggrecan and type Ⅱ collagen[17]. This suggests that the CRLF1/CLC complex may disrupt cartilage homeostasis and promote the progress of osteoarthritis.

Ectopic bone formation can be induced by injection of bone morphogenetic protein-2 (BMP-2) into the muscle. When gene expression was analyzed in such ectopically induced bone, expression of CRLF1 was induced 5-fold at day 5 after BMP-2 injection. Temporally, CRLF1 induction preceded the stage of chondrogenesis in this model of endochondral osteogenesis[18].

Based on the results described above two roles of CLRF1 can be inferred; mediation of immune response and regulation of autonomic nervous system. However, association of bone deformities with CLRF1 mutation in humans, as well as regulation of CRLF1 by TGF-β and its induction by BMP-2, suggests a role in formation of the extracellular matrix. There are no reports on expression of CRLF1 in the liver or its association with liver fibrosis. Here we describe the expression of CRLF1 in hepatic stellate cells and fibrotic livers and the effect of CLRF1 overexpression in the liver.

MATERIALS AND METHODS

Isolation and culture of hepatic stellate cells

Hepatic stellate cells (HSCs) were isolated by perfusion of rat livers with pronase and collagenase, followed by centrifugation over Nycodenz gradient, as described[19]. The purity of cells was estimated to be > 95% by desmin staining. The cells were cultured in uncoated plastic dishes in Dulbecco's modified Eagle's medium/10% fetal bovine serum for 2 d and used as quiescent HSCs or were cultured for 7 d and used as activated HSCs.

Induction of liver fibrosis

Liver fibrosis was induced in rats by intraperitoneal injections of carbon tetrachloride (CCl_4), 2 μL/g, twice a week for 4 wk. Livers were extracted, fixed in formalin and paraffin embedded sections were stained with Sirius red according to the standard procedure[20].

Adenovirus construction and injection

Adenovirus expressing human CRLF1 was constructed by cloning CRLF1 cDNA into pAdCMV TRACK vector and by subsequent recombination with pEasy vector in *Escherichia coli*, as described[21]. Viral particles were assembled and amplified in HEK 293 cells and purified by CsCl centrifugation, as described[22]. CRLF1 adenovirus also expressed green fluorescent protein (GFP) from an

Table 1 Primers used in reverse transcription-polymerase chain reaction analysis

CRLF1-r	5′primer	GGACAATCTGGTGTGTCACG
	3′primer	GGGCCCACAGTGTGATATTC
CLCF1-r	5′primer	TCCACCTTCCATCTGGTCTC
	3′primer	GCAGAGGCAAGCTAACATCC
CNTFR-r	5′primer	ACACCACGGCTATCACCTTC
	3′primer	ATTCGAGAGCTCCACATGCT
COL3A1-m	5′primer	ACGTAAGCACTGGTGGACAG
	3′primer	AGCTGCACATCAACGACATC
KC-m	5′primer	TCGCCAATGAGCTGCGCTGTC
	3′primer	GCTTCAGGGTCAAGGCAAGCC
IL-1β-m	5′primer	GCCCATCCTCTGTGACTCAT
	3′primer	AGGCCACAGGTATTTTGTCG
IL-6-m	5′primer	AGTTGCCTTCTTGGGACTGA
	3′primer	TCCACGATTTCCCAGAGAAC
TNF-α-m	5′primer	CGTCAGCCGATTTGCTATCT
	3′primer	CGGACTCCGCAAAGTCTAAG
Actin-m	5′primer	CGTGCGTGACATCAAAGAGAAGC
	3′primer	TGGATGCCACAGGATTCCATACC
Actin-r	5′primer	CGTGCGTGACATTAAAGAGAAGC
	3′primer	TGCATGCCACAGGATTCCATACC
COL1A1-r	5′primer	TGAGCCAGCAGATTGAGAAC
	3′primer	TGATGGCATCCAGGTTGCAG
COL1A2-r	5′primer	CTCACTCCTGAAGGCTCTAG
	3′primer	CTCCTAACCAGACATGCTTG
COL3A1-r	5′primer	AGGCCAATGGCAATGTAAAG
	3′primer	GGCCTTGCGTGTTTGATATT
FIB-r	5′primer	GAAAGGCAACCAGCAGAGTC
	3′primer	CTGGAGTCAAGCCAGACACA
α-SMA-r	5′primer	ACAGAGAGAAGATGACGCAG
	3′primer	GGAAGATGATGCAGCAGTAG
CRLF1-h	5′primer	CCAGAGAAACCCGTCAACAT
	3′primer	GCCTCCACCCAGATCTCATA

α-SMA: α-smooth muscle actin; CNTFR: Ciliary neurotrophic factor receptor; COL1A1: Collagen, type Ⅰ, α 1; COL1A2: Collagen, type Ⅰ, α 2; COL3A1: Collagen, type Ⅲ, α 1; CRLF1: Cytokine receptor like factor 1; CLCF1: Cardiotrophin-like cytokine factor 1; IL: Interleukin; TNF-α: Tumour necrosis factor-α; KC: Chemokine; FIB: Fibronectin; -m: Mouse; -r: Rat; -h: Human.

independent transcription unit, GFP served as a marker of viral transduction. Control virus expressed only GFP. Viruses were injected through tail vein into mice at a dose of 1×10^7 PFU/g. HSCs in culture for two days after isolation were transduced with the adenovirus at multiplicity of infection of 500 and culturing continued until day seven after isolation, when the cells were collected for analysis.

Reverse transcription-polymerase chain reaction analysis

Total RNA was isolated from HSCs using RNAeasy kit from Sigma. From livers, total RNA was isolated by homogenization of liver in 6 mol guanidine-isothiocyanate solution, followed by phenol/chlorophorm extraction and ethanol precipitation. Reverse transcription-polymerase chain reaction (RT-PCR) was performed by our well established procedure[23-26]. Briefly, reverse transcription was done with a primer that was specific for each individual RNA and subsequent PCR amplification was done in the presence of ^{32}P-dCTP to label the PCR products, which were resolved on a sequencing gel. The identity of products was confirmed by the expected size and by sequencing. To avoid saturation, the number of cycles in the PCR step was kept at a minimum, sufficient to give signals. The results obtained by this method are in excellent agreement with the results obtained by real time RT-PCR, as shown in our previous work[27]. The primers used are shown in Table 1.

Western blotting

Proteins were extracted from the liver by homogenization in Rippa buffer and 200 μg of total liver proteins was analyzed. For analysis of cellular medium, 100 μL of cellular medium collected from confluent HEK293 was directly loaded on the gel. Anti-CRLF1 antibody was purchased from Santa Cruz Biotechnology (sc-100297) and used at 1:1000 dilution.

RESULTS

HSCs are the main cells responsible for synthesis of extracellular matrix in liver fibrosis[28,29]. HSCs undergo activation from quiescent state into myofibroblasts upon a fibrogenic stimulus. Similar activation can be achieved by culturing HSCs *in vitro*[30]. To identify genes associated with activation of HSCs we used microarrays to compare the gene expression profile of quiescent rat HSCs and rat HSCs activated by culturing *in vitro*[31]. In two independent experiments we found that *CLRF1* gene was expressed dramatically higher in activated HSCs than in quiescent HSCs (Figure 1A). The genes encoding *LIFR*, *CNTFR*, *CTF1*, *IL6ST* and *CNTF* were constitutively expressed and showed no change in expression. Expression of CNTF was found to be only moderately increased in activated compared to quiescent HSCs using microarrays (Figure 1A). Expression of IL-6 was greatly increased in activated HSCs, but the level of IL6 receptor, which together with IL6ST and LIFR forms IL-6 receptor, was unchanged. This suggested that only IL-6 and CRLF1 are dramatically upregulated in the activation of HSCs, while the rest of the signaling machinery seems to be constitutively expressed.

To verify the microarray data we assessed the expression of *CLRF1*, *CLCF1* and *CNTFR* genes in quiescent and activated rat HSCs by RT-PCR (Figure 1B). In these experiments HSCs were only subjected to culture activation and were not stimulated with TGF-β or platelet-derived growth factor. We used our radio-labeled RT-PCR method which is in excellent agreement with the results obtained by real time RT-PCR, as demonstrated in our earlier work[27]. Expression of CNTFR and CLCF1 was similar in quiescent and activated HSCs and this result is in good agreement with the microarray data (Figure 1B). The expression of CRLF1 was dramatically increased in activated HSCs compared to quiescent HSCs, where it was undetectable (compare lanes 1 and 2). This is in excellent agreement with microarray data, which showed the logarithm of the signal ratio between activated and quiescent cells of 6 (Figure 1A). The results obtained by

A

Gene	Simbol	A *vs* Q signal log ratio	*P* value	A *vs* Q signal log ratio	*P* value
Cytokine receptor-like factor 1 (predicted)	CRLF1	6.3	0.00 002	6.5	0.00 002
Interleukin 6	IL-6	4.8	0.00 002	1.8	0.00 002
Cardiotrophin-like cytokine	CLCF1	2.5	0.0 000 023	1	0.000 088
Interleukin 6 signal transducer	IL6ST	1.4	0.00 002	0	0.5
Ciliary neurotrophic factor recepter	CNTFR1	1.4	0.5	-0.4	0.570 859
Cardiotrophin 1	CTF1	1.3	0.131 038	0	0.5
Interleukin 6 receptor	IL6R	0.9	0.000 078	-0.2	0.5
Leukemia inhibitory factor receptor	LIFR	0.2	0.003 919	0.4	0.5
Ciliary neurotrophic factor	CNTF	-0.3	0.980 376	-1	0.999 932
		Array Ⅰ		Array Ⅱ	

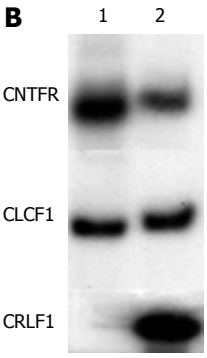

Figure 1 Genes upregulated in activation of hepatic stellate cells. A: Rat hepatic stellate cells (HSCs) were isolated and cultured for 2 d (quiescent HSCs) or for 7 d (activated HSCs) and total RNA was analyzed for gene expression by microarrays in two independent experiments. The fold increase in transcript level and *P* values for the selected genes is shown; B: Expression of cytokine receptor-like factor 1 (CRLF1), cardiotrophin-like cytokine factor 1 (CLCF1) and ciliary neurotrophic factor receptor (CNTFR) in quiescent and activated HSCs was estimated by reverse transcription-polymerase chain reaction. Q *vs* A: Quiescent HSCs vs activated HSCs; IL-6: Interleukin-6; IL6ST: Interleukin 6 signal transducer; IL6R: Interleukin 6 receptor; CNTF: Ciliary neurotrophic factor.

both of these techniques indicated that CRLF1 is dramatically upregulated during activation of HSCs.

To assess if CRLF1 expression is also upregulated in fibrotic livers we induced liver fibrosis by repeated injections of carbon tetrachloride CCl_4 into rats. Sirius red staining of one normal liver (N1) and two livers extracted from the CCl_4 treated animals are showed in Figure 2A. One of these livers showed a moderate degree of fibrosis (F1) and the other showed more massive bridging fibrosis (F4). Expression of CRLF1, CLCF1 and CNTFR mRNA in three normal livers, including the N1 liver (lanes 1-3), and five fibrotic livers, including the F1 and F4 livers (lanes 4-8) is showed in Figure 2B. CLCF1 was constitutively expressed in the liver and its expression did not change with induction of liver fibrosis. This is in agreement with its equal expression in quiescent and activated HSCs. Expression of CNTFR appeared to be slightly lower in fibrotic livers than in control livers (Figure 2B; compare lanes 4-8 to lanes 1-3), but CNTFR was clearly constitutively expressed. CRLF1 mRNA was detected at low levels in normal livers and its expression was increased in all fibrotic livers. There was a correlation between the degree of fibrosis and CRLF1 expression. For example, liver F1 had less fibrosis than liver F4 (Figure 2A) and expression of CRLF1 in liver F1 is lower than in liver F4. However, it is still higher than in all normal livers (Figure 2B). Thus, CRLF1 is again the only component that is upregulated in liver fibrosis, while CLCF1 and CNTFR are constitutively expressed. Since activation of HSCs is responsible for development of liver fibrosis and activated HSCs dramatically upregulate expression of CRLF1, it is likely that increased CRLF1 expression in fibrotic livers originates from the activated HSCs.

Since it was reported that CRLF1 can modulate immune response, we assess if overexpression of CRLF1 in the liver can induce changes in expression of proinflammatory genes. To achieve overexpression of CRLF1 in the liver we constructed an adenovirus expressing human CRLF1 and injected the adenovirus into tail vein of three mice. Human CRLF1 protein shares 95% identity to mouse CRLF1. Control adenovirus, expressing only GFP was injected into two mice. No liver fibrosis was induced in these animals, only overexpression of CRLF1 was achieved by adenoviral injections. Injection of adenovirus into circulation results in the clearance of the virus by the liver and transduction of all cell types in the liver. We measured the expression of CRLF1 protein (Figure 3A, upper panel) and mRNA (lower panel) in the livers of these animals two days after the viral injection. There was high level of expression in all three CRLF1 adenovirus injected animals. There was also a very good correlation between expression of CRLF1 protein and mRNA. This was in sharp contrast to CRLF1 expression in control livers, which was detect at very low levels. Next, we examined if high CRLF1 level in the liver can stimulate expression of four proinflammatory cytokines. We chose chemokine (KC) (mouse homolog of gro-α)[32,33], IL-1, IL-6 and TNF-α, as the most common cytokines. Expression of KC, IL-1β, IL-6 and TNF-α showed some variation between the animals but neither cytokine was upregulated in the CRLF1 overexpressing livers (Figure 3). The measurement of aminotransferases in plasma of these animals showed normal levels (not shown). From these results we concluded that high levels of CRLF1 in the liver do not stimulate production of proinflammatory cytokines nor they cause liver damage.

Activated HSCs express high level of CRLF1, while its expression in quiescent HSCs is undetectable (Figure 1). However, quiescent HSCs express CLCF1 and CNTFR, therefore they may be responsive to CRLF1 stimulation. We decided to overexpress CRLF1 in quiescent HSCs and assess what effects it may have on activation of HSCs and collagen expression. To verify that adenovirus transduced cells can secrete CRLF1 we infected HEK293 cells and measured the CRLF1 in the cellular medium by western blot. High level of secreted CRLF1 was found in the medium of CRLF1 adenovirus transduced cells, while it was undetectable in control cells

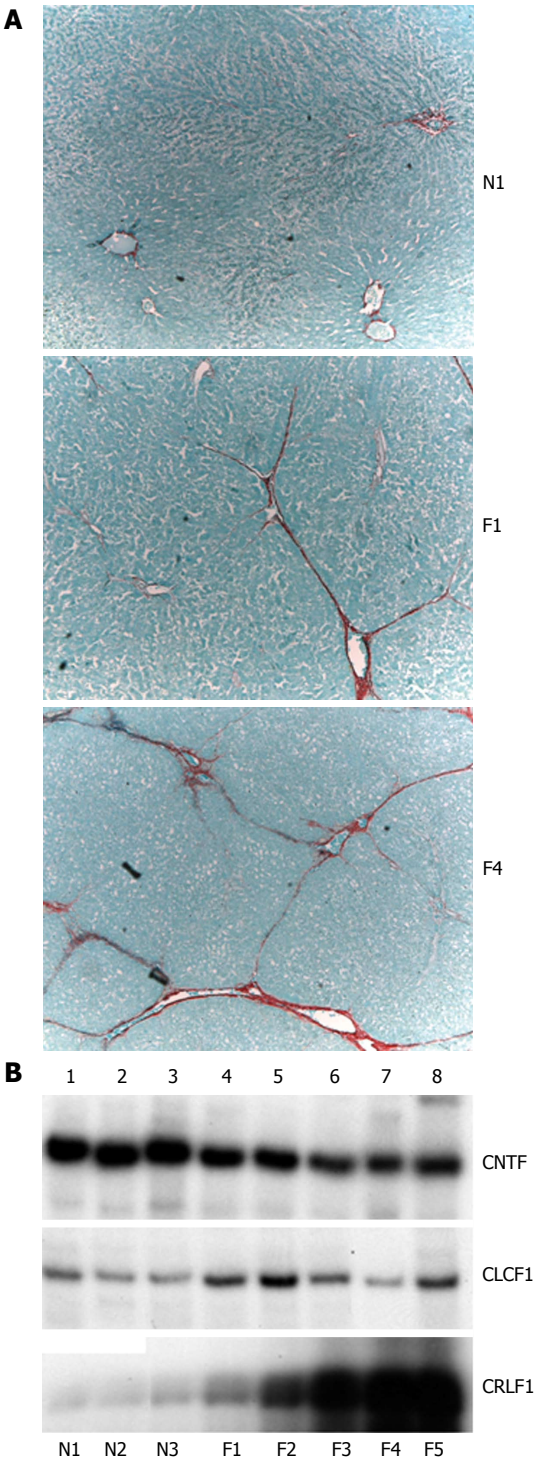

Figure 2 Expression of cytokine receptor-like factor 1 in fibrotic livers. A: Sirius red staining of liver slices from three representative animals; N1: Normal liver; F1: Liver with moderate degree of fibrosis induced by CCl_4; F4: Liver with high degree of fibrosis induced by CCl_4; B: Reverse transcription-polymerase chain reaction analysis of expression of cytokine receptor-like factor 1 (CRLF1), cardiotrophin-like cytokine factor 1 (CLCF1) in three normal livers (N1-N3) and five fibrotic livers (F1-F5).

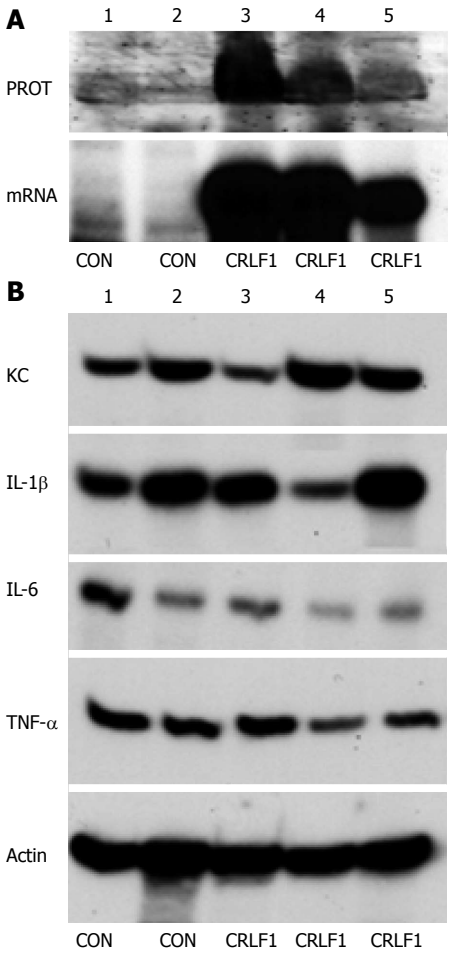

Figure 3 Overexpression of cytokine receptor-like factor 1 in normal liver does not increase expression of profibrotic cytokines. A: Overexpression of cytokine receptor-like factor 1 (CRLF1). Control adenovirus was injected into tail vein of two mice and adenovirus expressing cardiotrophin-like cytokine factor 1 (*CLCF1*) gene was injected into three mice. After three days liver proteins and RNA were extracted and expression of CLRF1 protein was analyzed by Western blot (top panel) and CLRF1 mRNA was analyzed by reverse transcription-polymerase chain reaction (RT-PCR) (bottom panel); B: Expression of profibrotic cytokines. Expression of the indicated profibrotic cytokines was analyzed by RT-PCR in the control (CON) and CRLF1 overexpressing livers. PROT: Protein; CNTFR: Ciliary neurotrophic factor receptor; IL: Interleukin; TNF-α: Tumour necrosis factor-α; KC: Chemokine.

(Figure 4A). This verified that transduction of HSCs will result in production of secreted CRLF1 capable of stimulating its receptor. Therefore, we transduced quiescent primary rat HSCs with CRLF1 adenovirus two days after isolation, when they still have a quiescent phenotype[31] (Figure 1). We then analyzed the expression of α-smooth muscle actin (α-SMA), as the marker of activation[34], at day 6 (4 d after the viral transduction). The RT-PCR analysis (Figure 4B) revealed that expression of α-SMA was reduced in HSCs ectopically expressing CRLF1. This suggested that overexpression of CRLF1 in quiescent HSCs can slow down the activation process of isolated HSCs in culture. We also measured expression of collagen α1 (I) and α2 (I) mRNAs, encoding type I collagen, and collagen α1(Ⅲ) mRNA, encoding type Ⅲ collagen (Figuer 4B). There was a decrease in expression of collagen α1 (I) and α2 (I) mRNAs, which correlated with lower expression of α-SMA. This, again, suggested a retarded activation process of HSCs. The expression of collagen α1 (Ⅲ) mRNA seemed to be

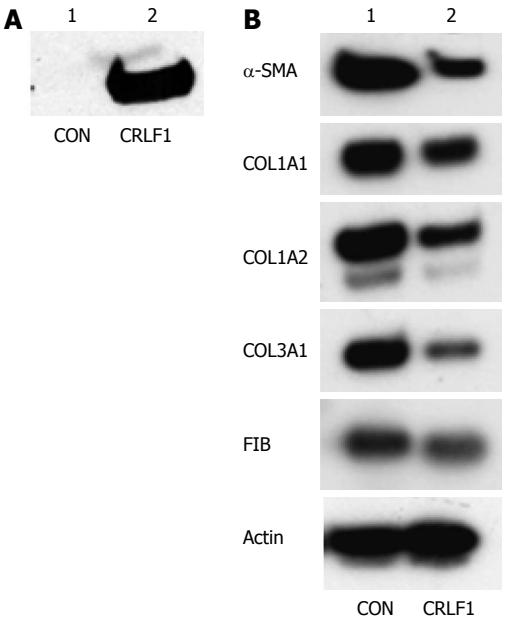

Figure 4 Cytokine receptor-like factor 1 decreases expression of type Ⅲ collagen by hepatic stellate cells. A. Expression of adenovirus delivered cytokine receptor-like factor 1 (CRLF1) in the cellular medium. HEK293 cells were transduced with control or CRLF1 expressing adenovirus. One day after transduction, CRLF1 protein was measured in the cellular medium by Western blotting; B: Stimulation of hepatic stellate cells (HSCs) with CRLF1. HSCs at day 2 after isolation were transduced with control or CRLF1 adenovirus. At day 7, expression of α-smooth muscle actin (α-SMA), collagen α1 (Ⅰ), collagen α2 (Ⅰ), collagen α1 (Ⅲ) and fibronectin (FIB) mRNAs was analyzed by reverse transcription-polymerase chain reaction. CON: Control; COL1A1: Collagen, type Ⅰ, α1; COL1A2: Collagen, type Ⅰ, α2; COL3A1: Collagen, type Ⅲ, α1.

more downregulated than that of collagen α1 (Ⅰ) and α2 (Ⅰ) mRNAs. This indicates that CRLF1, in addition to retarding activation of HSCs, may specifically inhibit the expression of collagen type Ⅲ.

To assess if the effect on expression of type Ⅲ collagen can be reproduced in the whole liver, we analyzed expression of collagen α1 (Ⅲ) mRNA in two livers of mice injected with CRLF1 adenovirus. Again, no liver fibrosis was induced, only adenoviruses were injected. As shown in Figure 5, collagen α1 (Ⅲ) mRNA was expressed at lower level in CRLF1 overexpressing livers than in control livers, suggesting that CRLF1 can negatively regulate collagen type Ⅲ expression in the whole liver. Expression of collagen α1 (Ⅰ) and α2 (Ⅰ) mRNAs was very low in control livers and we could not detect any decrease in CRLF1 livers (not shown). From these experiments we concluded that CRLF1 may control the activation of HSCs and specifically regulate expression of type Ⅲ collagen in the liver and HSCs.

DISCUSSION

This is the first report on expression of CRLF1 in liver fibrosis and in HSCs. Humans with the mutations in *CRLF1* gene suffer from cold sweat syndrome or Crisponi syndrome[9,10,12-14]. While these syndromes led to conclusion that CRLF1 is required for development

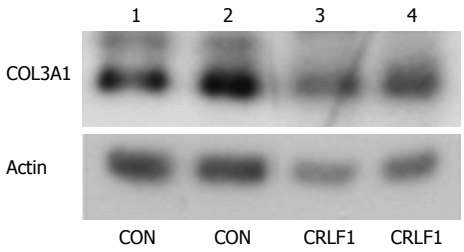

Figure 5 Cytokine receptor-like factor 1 decreases type Ⅲ collagen expression in the whole liver. RNA was extracted from two control livers and two livers that overexpressed cytokine receptor-like factor 1 (CRLF1) after adenoviral delivery and expression of collagen α1 (Ⅲ) mRNA was analyzed by reverse transcription-polymerase chain reaction. CON: Control; COL3A1: Collagen, type Ⅲ, α1.

of peripheral nervous system, the craniofacial malformations of these patients suggest that CRLF1 may be important for proper formation of extracellular matrix. HSCs are the primary cells responsible for secretion of extracellular matrix in liver fibrosis. Upon fibrogenic stimulus HSCs undergo activation and dramatically upregulate synthesis of collagens type I and type Ⅲ[26]. Type I collagen forms large diameter fibrils and is the major component of the sca[35], while type Ⅲ collagen forms fibrils of low diameter and is the main component of scar less wound healing[36]. Thus, the balance between the amount of type I and type Ⅲ collagen can determine the extent and irreversibility of scarring.

We found that quiescent HSCs express CRLF1 at undetectable levels while its expression in activated HSCs is dramatically increased. Other proteins that participate in CRLF1 signaling, CLCF1 and CNTFR1, are constitutively expressed, therefore, it is highly likely that CRLF1 is the regulatory component of the signaling system in HSCs. CRLF1 is expressed at low levels in normal livers, however, its expression correlates with the degree of liver fibrosis and is dramatically increased in highly fibrotic livers. This suggests that it originates from activated HSCs. It has been reported that CRLF1 expression is activated by TGF-β[17], which is the main profibrotic cytokine[37,38]. Thus, TGF-β may not only stimulate HSCs to transdifferentiate into myofibroblasts, but it may be responsible for upregulation of CRLF1 production. Other liver cells are likely to produce small amounts of CRLF1, as evidenced by low level of expression in normal livers that do not contain activated HSCs.

High CRLF1 expression in the liver does not stimulate expression of proinflammatory cytokines. This was evidenced by adenoviral delivery of CRLF1 into normal livers. We did not observe an increase in expression of TNF-α, IL-1β or KC chemokine. There was also no evidence of increased hepatocellular damage, as assessed by measuring aspartate aminotransferase and alanine aminotransferase in plasma (not shown). Thus, expression of CRLF1 alone is not sufficient to induce proinflammatory changes in the liver.

Overexpression of CRLF1 in quiescent HSCs attenuated their activation in culture. When CRLF1 was

ectopically expressed in isolated quiescent HSCs, the expression of the marker of transactivation into myofibroblasts, α-SMA[31,34], was decreased. The expression of collagens type I and Ⅲ was also attenuated. The effect on multiple markers of activation suggested the general effect on this process. How CRLF1 may slow down culture activation of HSCs is not clear. Since CLCF1, CNTFR1, LIFR and IL6ST are constitutively expressed in HSCs, this may involve activation of this signaling pathway by the ectopical CRLF1. What downstream effectors of the pathway may be involved is not clear, but it is important to elucidate this, because it may shed light on the overall mechanism of HSCs activation.

The expression of collagen α1 (Ⅲ) mRNA in HSCs overexpressing CLRF1 was decreased more than that of collagen α1 (Ⅰ) and α2 (Ⅰ) mRNAs. This suggested that CLRF1 can specifically downregulate type Ⅲ collagen, in addition to inhibiting HSCs activation. This was confirmed in the whole liver. Type Ⅲ collagen was easily detectable in normal livers by RT-PCR, but when CLRF1 was overexpressed in two livers by adenoviral delivery, its expression was significantly decreased. The mechanism by which CRLF1 affects expression of type Ⅲ collagen in the liver is not known. It may involve association of CRLF1 with CLCF1 and their binding to CNTFR subunit of the receptor, because these components are constitutively expressed. The receptor activates suppressor of cytokine signaling (SOCS) family of transcription factor, in particular SOCS3, which is translocated into the nucleus and regulates transcription of its responsive genes[39]. However, it is not known if collagen α1 (Ⅲ) gene can be directly or indirectly regulated by SOCS transcription factors.

What is the consequence of high CRLF1 expression in fibrotic livers? CRLF1 alone can not stimulate inflammation in the liver, although there is a possibility that it may potentiate the action of other cytokines. This, however, remains to be elucidated. CRLF1 seems to attenuate activation of HSCs *in vitro* and its expression is proportional to the degree of fibrosis *in vivo*, so it may regulate activation of HSCs in the liver. In addition, it may suppress expression of type Ⅲ collagen. This would favor deposition of type I collagen over type Ⅲ collagen, what was observed in fibrotic livers[40] and in hypertrophic scarring[41]. In osteoarthritic cartilage, it was proposed that CRLF1 suppresses expression of type Ⅱ collagen[17]. So, altering extracellular matrix by changing the balance between different collagens may be a general property of CRLF1.

In conclusion, we show that CRLF1 is dramatically upregulated in liver fibrosis and in activated HSCs. Overexpression of CRLF1 does not stimulate expression of proinflammatory cytokines, but inhibits activation of HSCs *in vitro* and reduces expression of type Ⅲ collagen in the whole liver. By affecting the composition of extracellular matrix and activation of HSCs, CRLF1 may modulate the extent of fibrosis.

COMMENTS

Background

Hepatic stellate cells (HSCs) are the main cells responsible for development of liver fibrosis. In fibrosis, HSCs undergo activation from their quiescent state into myofibroblasts and dramatically upregulate expression of type Ⅰ and type Ⅲ collagens. Multiple cytokines have been implicated in this process, with transforming growth factor-β being the most potent profibrotic cytokine. The role of other cytokines in regulation of collagen expression is less well understood.

Research frontiers

Cardiotrophin-like cytokine factor 1 (CLCF1) is a cytokine implicated in development of central nervous system and is mutated in patients with cold sweat syndrome and Crisponi syndrome. Cytokine receptor-like factor 1 (CRLF1) is upregulated in damaged osteoarthritic cartilage. It acts as a dimer with CLCF1 by activating ciliary neurotrophic factor receptor (CNTFR). CNTFR in complex with two additional subunits, interleukin 6 signal transducer (IL6ST) and LIFR, signals by triggering STAT/ERK signal transduction pathway. CNTFR does not bind IL-6, although it shares IL6ST and LIFR subunits with IL-6 receptor. It is unclear how CRLF1/CLCF1/CNTFR activity regulates development of neurons or contributes to osteoarthritis and the rare congenital syndromes. There have been no reports on expression or a role of CRLF1 in fibrosis.

Innovations and breakthroughs

This is the first report on expression of CRLF1 in HSCs and liver fibrosis. CRLF1 is dramatically upregulated in activation of HSCs *in vitro* and *in vivo*. The other two specific components of the signaling pathway, CLCF1 and CNTFR, are constitutively expressed and do not change in fibrosis. This suggests that upregulation of CRLF1 triggers the pathway, activation of which contributes to the pathogenesis of fibrosis. A novel finding is also that CRLF1 decreases expression of type Ⅲ collagen. By changing the ratio of different collagens in the extracellular matrix, CRLF1 may promote fibrilogenesis. Thus, this work provided a new insight into molecular mechanisms of HSCs activation and fibrosis development.

Applications

By discovery of the role of CRLF1 in hepatic fibrosis, a possibility of new therapeutic intervention is suggested. CRLF1 is a secreted cytokine receptor-like molecule and by designing agonists or antagonists of its interaction with CLCF1 or CNTFR a novel strategy to modulate liver fibrosis may be initiated.

Terminology

CRLF1 is a 422 amino-acids secreted protein that represents a novel class of soluble cytokine receptor-like molecules. Its ligand is CLCF1 and the soluble CRLF1/CLCF1 dimer binds the membrane anchored receptor CNTFR. CNTFR can also bind another ligand, ciliary neurotrophic factor (CNTF) and a competition between CRLF1/CLCF1 dimer and CNTF for the CNTFR binding has been demonstrated. CNTFR is a non-signaling component of the heterotrimeric transmembrane complex composed of CNTFR, IL6ST and LIFR. The IL6ST and LIFR subunits of this heterotrimeric receptor are also components of IL-6 receptor. However, CNTFR does not bind interleukin-6.

Peer review

The authors provided evidence that expression of CRLF1 is highly upregulated in activation of HSCs, as well as in fibrotic livers. In the liver its expression is correlated with the degree of fibrosis. Interestingly, the other components of the signaling pathway, CLCF1 and CNTFR, are constitutively expressed in HSCs and livers. Therefore, CRLF1 regulates the activity of the pathway in fibrosis. CRLF1 attenuates the expression of type Ⅲ collagen. This may change the ratio of type Ⅰ to type Ⅲ collagen in the extracellular matrix, affecting its polymerization, crosslinking and turn over. This is the first report on the expression and effects of CRLF1 in fibrosis and represents a significant contribution to the understanding of the molecular mechanisms of hepatic fibrosis.

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ORIGINAL ARTICLE

Prevalence of HIV and HCV infections in two populations of Malian women and serological assays performances

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Abstract

AIM: To estimate the prevalence of human immunodeficiency virus (HIV) and hepatitis C virus (HCV) infections in women in Mali and to evaluate the performance of serological assays.

METHODS: Two prospective studies were conducted in 2009 and 2010 in Mali. They concerned first, 1000 pregnant women attending six reference health centers in Bamako (Malian capital) between May 26 and June 16, 2009; and secondly, 231 women over 50 years who consulted general practitioners of two hospitals in Bamako between October 25 and December 24, 2010. Blood samples were collected and kept frozen in good condition before analysis. All samples depicted as positive using HIV/HCV enzyme immuno-assay screening assays were submitted to confirmation analysis. Molecular markers of HCV were characterized.

RESULTS: The seroprevalence of HIV and HCV in the population of pregnant women was 4.1% and 0.2% respectively. Among older women the seroprevalence was higher and similar for HIV and HCV (6.1% *vs* 6.5%). The anti-HIV prevalence was not different in young and older women (4.1% *vs* 6.1%). In contrast, the anti-HCV prevalence was higher in older compared to younger women (6.5% *vs* 0.2%, $P < 0.01$). Of 2 pregnant women who were HCV seropositive, only one was polymerase chain reaction (PCR) reactive and infected by genotype 2, with a viral load of 1600 IU/mL. Regarding older women who were HCV seropositive, 13 out of 15 were PCR reactive, infected by genotype 1 or 2. Globally HCV genotype 2 was predominant. The positive predictive value (PPV) measured with VIKIA HIV test in young women was 100% therefore significantly higher than the 87.5% measured in older women ($P < 0.05$). Conversely, the PPV measured with Monolisa HCV assay in older women was 88.2% and higher than the 14.3% measured in younger women ($P < 0.01$).

CONCLUSION: Whereas HIV prevalence was similar in both subpopulations HCV was more frequent among older women ($P < 0.01$). The PPV of screening assays varied with the age of the subjects.

Key words: Human immunodeficiency virus; Hepatitis C virus; Serology; Molecular diagnostics; Women; West Africa; Bamako

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INTRODUCTION

Discovered in 1989 by Houghton and coworkers, the hepatitis C virus (HCV) is the leading cause of chronic hepatitis and cirrhosis in Europe and North America[1]. In these countries, before the introduction of preventive measures such as blood donor selection and screening of blood donations, blood transfusion was largely responsible for the transmission of HCV, and amounted for up to 1/3 of cases[1].

The estimation of HCV prevalence in the general population is imprecise because of the difficulty in collecting representative samples and the cost of such studies[1].

In 1999, WHO estimated that about 3% of the world population was infected with hepatitis C and that at least 170 million chronic carriers of the virus were at risk of complications of developing cirrhosis and hepatocellular carcinoma, including more than 5 million in Europe[1,2]. HCV transmission is mainly parenteral[3]. Vertical transmission (mother-to-child) of HCV is estimated to be less than 5%, but in case of human immunodeficiency virus (HIV) co-infection, the risk of mother-to-child transmission can reach 15% to 20%[4]. Similarly, HCV/HIV co-infection promotes the progression of hepatitis to cirrhosis[5]. In sub-Saharan Africa, the prevalence of HCV infection varies between 0.1% and 13.8 %[6].

As for Malian blood donors, HCV seroprevalence was reported at 3.30%[7]. In Mali, the seroprevalence of HCV is not well elucidated in the population of pregnant women, and even less in the general population. Molecular epidemiology of HCV is also unknown. In this country, while prevention and treatment programs are implemented for HIV, much remains to be done for viral hepatitis in general.

In the literature there are controversies about the performance of HCV enzyme immuno-assay (EIA) tests in sub-Saharan Africa. This has stimulated scientific interest to document HCV epidemiology as well as the performance of HCV EIA tests among women in Mali in order to guide public health decisions. According to UNAIDS epidemic update in 2007 an estimated 22.5 million (20.9-24.3 million) people living with HIV, or 68% of the world cases, are in sub-Saharan Africa[8]. West Africa, considered as the most populated region of this continent, has a postulated HIV prevalence between 1% and 5%[9]. This region is the epicenter of the HIV-2 epidemic[10].

HIV prevalence is 1.3% in the general population in Mali (EDSM-IV 2006, final report 2007)[11]. In Mali, to prevent HIV mother to child transmission few rapid tests exist for the screening of pregnant women who attend health centers for antenatal care. The VIKIA HIV 1/2 3rd generation test (BioMerieux) is both highly sensitive and specific, does not require complex equipment and therefore could facilitate HIV testing especially in poorer areas[12,13]. This prompted us to evaluate the effectiveness of this test in Malian women.

Our study was conducted in Bamako (Malian capital). Mali is a country located in West Africa which borders with Ivory Cost and Guinea Conakry to the South, Mauritania and Senegal to the West, Algeria to the North, Niger to the East and Burkina Faso to the South-East (Figure 1). Mali extends to 1 240 192 square kilometers and comprises a population estimated at 13 415 205 inhabitants, among which 8 649 035 live in rural areas and 4 766 170 in urban areas[14]. The choice of Bamako was warranted by the fact that this city achieved the maximum coverage rate of antenatal care (ANC) between 2006 and 2007, estimated at 85%-90%, with an ANC assiduity index at 2.12 to 2.21[15].

To estimate and compare the prevalence of both infections and evaluate HCV EIA performance, we undertook a study in two populations of women in Mali: (1) Pregnant women (or young women): this population, although exclusively female, is fairly representative of the general population because the patients are not selected on specific risk factors and are supposedly healthy[16]. Furthermore, these women can be easily contacted and represent the vector of the major modes of HIV transmission (sexual and vertical); (2) Women over 50 years attending general practice in two hospitals in Bamako: this second population is likely to be better informed on the epidemiology of HCV, as suggested by Ndong-Atome *et al*[17] who described an association between age and HCV seroprevalence.

MATERIALS AND METHODS

Subjects

Initially, this was a prospective study including pregnant women attending ANC in the six Health Centers of Reference in Bamako. Exclusion criteria were: refusal to participate in the study and treatment with heparin (an inhibitor of PCR). In a second step, the study was extended to women over 50 years attending general practice in two hospitals (CHU Gabriel Touré, and Hospital Mother and Child) in Bamako. The exclusion criteria were refusal, any physical or mental condition precluding investigation as well as treatment by heparin.

This study has been carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association. The study was approved ethically under the reference number 08-0006/INRSP-CE (Ethical Committee of National Institute of Public Health) of Mali. All participating subjects remained anonymous and gave voluntary informed consent.

Figure 1 Map of Mali with neighboring countries. Figure obtained from website: www.geography.about.com.

Sample collection

Standard operating procedures were issued to control blood collection, preservation and transport. Physicians, researchers, midwives and laboratory technicians were trained in the application of procedures. Sample transport by drivers was supervised.

Sample treatment

A sample of 15 mL of venous blood was collected in vacutainer tubes [(dry and ethylenediaminetetraacetic acid (EDTA)] from all pregnant women in the study. From these primary samples serum and plasma were separated by refrigerated centrifugation (5 ℃) at 1500 rpm for 10 min. For women > 50 years, the samples were taken only in EDTA vacutainer tubes (10 mL/participant). The plasma was separated by centrifugation at 5000 rpm for maximum 5 min at room temperature. A delay of maximum 6 h between blood collection and freezing (-20 ℃ to -80 ℃) was always respected by the strategy put in place to ensure the quality of the collection samples. A large number of aliquots (6 × 1 mL/pregnant woman; 4 × 1 mL/older woman) were harvested before analysis in order to avoid multiple freeze-thaw cycles.

Serological methods

All assays were performed according to the manufacturer's instructions. The samples were analyzed after a single thaw. All tests were performed according to procedures accredited to EN15189 norm in the AIDS Reference Laboratory of the CHU-ULg.

Screening and confirmation of HIV infection: The rapid test VIKIA HIV1/2 (BioMérieux), a 3rd generation assay based on the principle of immunochromatography (ICT or lateral flow), known for its sensitivity and specificity[12] was used as first line test.

The INNO-LIA HIVI/Ⅱ score based on the line immuno assay (LIA) principle was used to confirm positive results obtained with the first-line tests and to distinguish HIV-1 from HIV-2 infections (Innogenetics Gent, Belgium). The undetermined samples were tested with VIDAS DUO assay to search for HIV p24 Ag.

Screening and confirmation of HCV infection: (1) First-line test. For pregnant women, two EIA tests were used: Innotest HCV Ab Ⅳ (Innogenetics, Belgium), which detects HCV antibodies; Monolisa HCV Ag/Ab Ultra (Biorad, Belgium), which detects simultaneously HCV antibody and antigen. For women over 50 years, the combined test (Monolisa) was solely used as a screening test. Samples found positive in a first run were further analyzed in two additional replicates. Samples giving at least two positive replicates (test ratio ⩾ 1) out of three with the screening test were considered "positive". Samples which were positive in the first run but whose result was not reproduced at least once were considered "negative"; and (2) Second-line serological assay. INNO-LIA HCV (Innogenetics) was used to confirm samples found positive with the screening test. All series of tests were validated by a specific internal quality control material (Pelispy, Westburg). The test ratio value of internal quality control was encoded in MedLab QC software for run validation.

Molecular biology

For pregnant women: HCV RNA was assayed using qualitative PCR (COBAS AMPLICOR hepatitis C, Roche Laboratory). Samples containing HCV RNA were further analyzed by quantitative PCR (COBAS AMPLICOR HCV MONITOR, Roche Laboratory).

For women over 50 years: HCV RNA was quantified using HCV m2000 Real Time PCR kit (Abbott) which became available for this part of the study. All samples tested positive by the combined test (Monolisa) but further found LIA negative, thus potentially indicative of very early infections, were submitted to PCR analysis. The infecting genotype was defined by the use of Versant HCV Genotype Assay test LiPA of Siemens Healthcare Diagnostics.

Statistical analysis

Results are presented as mean ± SD (range) for continuous variables and as frequencies (%) for categorical variables. Comparisons of categorical variables between groups were done using a χ^2 test. Positive predictive values (PPV) were calculated in each group. Results were considered to be significant at the 5% level ($P < 0.05$). Calculations were done using SAS version 9.3 for Windows (SAS Institute, Cary, NC, United States).

RESULTS

Prevalence of HIV and HCV infections in pregnant women

In the pregnant women population ($n = 1000$), age ranged from 14 to 50 years; with a mean of 25.2 ± 6.3 years.

Prevalence of HIV: Out of 1000 pregnant women tested, 41 were confirmed HIV seropositive among 45 subjects with a positive screening assay; the rate of indeterminate results (VIKIA+, LIA Ind) was 8.9% (4/45).

Table 1 Hepatitis C virus serological and biomolecular profile of 2 positive pregnant women

Subject	MONOLISA-HCV (test ratio value)			INNOTEST-HCV (test ratio value)			LIA-HCV (reactivity score)						LIA-HCV	PCR HCV	Genotype HCV
	X1	X2	X3	X1	X2	X3	C1	C2	E2	NS3	NS4	NS5			
AD3	5.84	6.12	6.08	5.50	5.71	5.58	4	1	0	3	2	0	Pos	Pos	2
OM4	0.84	0.28	0.27	3.32	2.54	2.38	1	0	0	0	1	0	Pos	Neg	-

AD3 and OM4: Initials of pregnant women from reference health center (municipality No. 3 and No. 4); X1: Test ratio value for initial essay; X2, X3: Test ratio value for replicate assays; C1 and C2: Core protein 1 and 2 of hepatitis C virus (HCV); E2: Transmembrane protein of HCV; NS3, NS4 and NS5: Non-structural proteins of HCV. PCR: Polymerase chain reaction.

A unique profile was found for these 4 indeterminate results due to an isolated gp41 band with intensity between 0.5+ and 2+. These 4 undetermined were tested negative with VIDAS DUO. The rate of false positive results (VIKIA+, LIA-) was 0%. The HIV seroprevalence in this population was 4.1% (95%CI: 2.90%-5.30%). Among the confirmed positive samples, HIV-1 species represented 95.1% (39 HIV-1 and 2 HIV-2).

Prevalence of HCV: Two pregnant women out of 1000 (Table 1) were confirmed HCV positive, with one single proven case of active infection (HCV-RNA pos, 1600 IU/mL, genotype 2). The rates of samples found reproducibly positive with HCV EIA tests but confirmed negative by LIA (false positives) did not differ for INNOTEST (25/45; 55.6%) or MONOLISA (6/14; 42.9%) ($P > 0.05$).

The OM4 sample giving an initial discrepant result between the two tests EIA HCV (Monolisa- Innotest+), was confirmed positive by LIA analysis (but with only two bands of weak intensity) but negative by PCR. The same sample analyzed in triplicates was found repeatedly negative with the Monolisa test. Based on the results of HCV LIA, the HCV seroprevalence measured in this population of young Malian women was very low: 0.2% (95%CI: 0.0%-0.4%).

Prevalence of HIV and HCV infections in women over 50 years old

In the 231 older women, ages ranged from 51 to 89 years; with a mean of 62.1 ± 8.6 years.

Prevalence of HIV: Out of 231 subjects, 14 were confirmed HIV seropositive among 19 found positive with the VIKIA test. The rate of indeterminate results (VIKIA+, LIA Ind) was 15.8%, i.e., 3 samples out of 19. Among these 3 samples, one was found with an isolated HIV1 gp41 band (at an intensity of 2+) and an isolated HIV2 gp36 band (at an intensity of 1+); in the 2 others, an isolated HIV1 gp41 band was detected at an intensity of 1+ and 2+, respectively. The rate of false positive results (VIKIA+, LIA-) was 10.5% (2/19). All indeterminate (3/3) and false positive (2/2) samples tested negative with VIDAS DUO HIV assay.

HIV seroprevalence in this population was 6.06% (95%CI: 2.96%-9.16%). Among the confirmed positive samples, HIV-1 species represented 64.3% (9 HIV-1 and 5 HIV-2). There was significantly more HIV-2 in women > 50 years (5/14) than in young females (2/41), $P < 0.01$.

Prevalence of HCV: Out of 231 subjects, 14 were confirmed seropositive out of 21 found reproducibly positive with the Monolisa assay. One sample showing an isolated HCV NS3 band (with an intensity of 4+) was found PCR positive. Taking this sample into account, the HCV seroprevalence measured in this population of older women was 6.49% (15/231) (95%CI: 3.31%-9.67%).

Viral HCV-RNA was detected in 13/15 seropositive women. Among them, 12 cases of active infection with genotypes 1 or 2 and viral loads ranging from 20 503 to 12 352 743 IU/mL were recorded. In one case, the viral load was < 12 IU/mL. Infection with HCV genotype 2 was much more represented, i.e., 10/12 (83.3%).

In this older population the rate of HCV false positives by ELISA was 2/21 (9.5%). This rate was significantly different from that of 6/14 (42.9%) measured in the younger population ($P < 0.05$). The HCV test algorithm and main results are depicted in Figure 2.

Comparison of HIV and HCV prevalence in young and older women

Considering the overall population of both young and older women, the estimated seroprevalences were 4.5% and 1.3% for HIV and HCV, respectively. The rate of active HCV infection was 1.1%.

Whereas the prevalence of HIV infection was not significantly different in the two series, the HCV seroprevalence was significantly higher in older women as was also the prevalence of HCV-RNA ($P < 0.01$). Among 13 HCV genotypes identified at all, there were 11 HCV genotype 2 (Table 2).

In young women, HIV infection was more common than HCV infection while HCV infection was more frequent in older women. The proportion of HIV1 was equal in both subpopulations, but HIV2 was more common in older women compared to pregnant ones. HCV genotype 2 was more frequent in older women (Figure 3).

Absence of HIV-HCV co-infection

Regardless of the two series, no HIV/HCV co-infection was detected but in each population, one case of "co-indeterminate" HCV/HIV results was found. The serum of a young woman initially depicted as doubtful and then negative using VIKIA test was analysed with LIA HIV

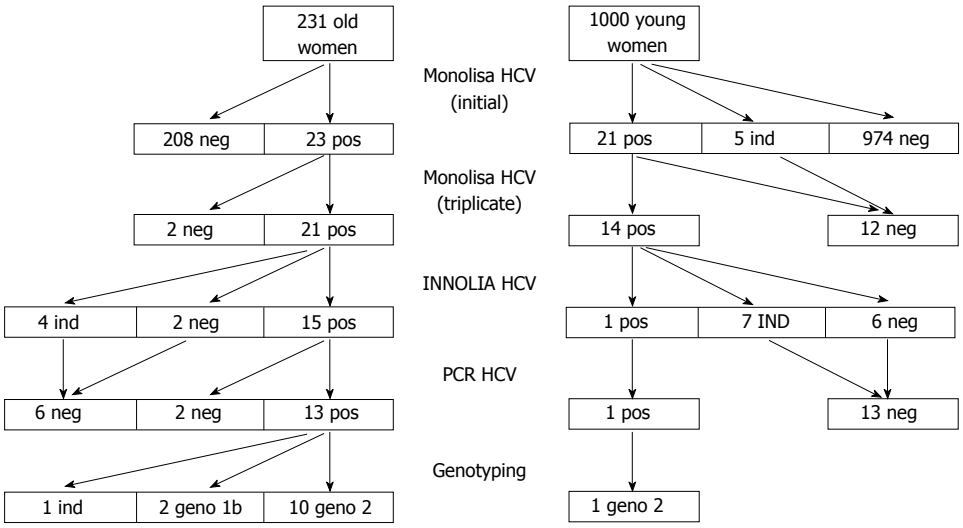

Figure 2 Algorithm of hepatitis C virus testing in the two female populations. Monolisa hepatitis C virus (HCV) (initial): Samples tested initially with Monolisa and results observed; Monolisa HCV (triplicate): Samples positive with initial test and found at least once positive in two additional replicates (step of duplicate is not represented here); INNOLIA HCV: Monolisa positive samples analyzed by INNO-LIA HCV confirmation test; PCR HCV: INNO-LIA HCV tested samples further analyzed by polymerase chain reaction (PCR); Genotyping: Positive PCR samples analyzed for genotype determination; ind: Doubtful result from Monolisa test; IND: Indeterminate result from INNO-LIA HCV confirmation test; Geno 1b: HCV-1 subtype b genotype; Geno 2: HCV-2 genotype.

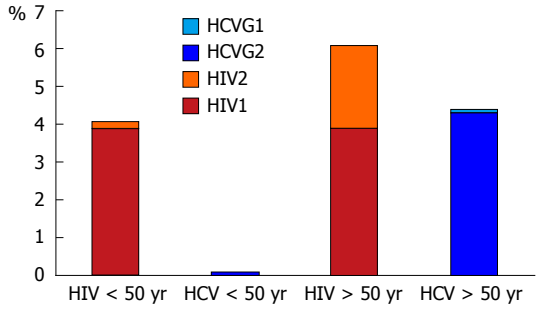

Figure 3 Prevalences of human immunodeficiency virus and hepatitis C virus compared according to age. Human immunodeficiency virus (HIV) < 50 years old: Young women with HIV infection; HIV > 50 years old: Older women with HIV infection; Hepatitis C virus (HCV) < 50 years old: Young women with HCV infection; HCV > 50 years old: Older women with HCV infection; HCVG1: HCV genotype 1; HCVG2: HCV genotype 2; %: Prevalence.

Table 2 Human immunodeficiency virus and hepatitis C virus seroprevalences, hepatitis C virus-RNA and genotype in the 2 series

Series	*n*	HIV Ab	HCV Ab	PCR-HCV	HCV genotype		
		%	%	%	1	2	other
Series 1 W ≤ 50 yr old	1000	4.10	0.20	0.10	0	1	0
Series 2 W > 50 yr old	231	6.06	6.49	5.60	2	10	0

Series 1 (W ≤ 50 years old): Young women population; Series 2 (W > 50 years old): Older women; HIV Ab: Serological markers of human immunodeficiency virus (HIV) (or anti-HIV); HCV Ab: Serological markers of hepatitis C virus (HCV) (or anti-HCV); PCR HCV: Virological markers of HCV [polymerase chain reaction (PCR) reactive results, or RNA detection]; %: Prevalence.

for confirmation. It showed a co-indetermination with an isolated p17 HIV band (at an intensity of 2+) and an isolated band of low intensity (1+) for the HCV C1 protein. The sample of this young woman was found HIV negative with VIDAS DUO test. In the older women series, one sample showed an isolated NS3 HCV band with low intensity (0.5+) and an isolated gp41 HIV band (with an intensity of 2+).

Technical considerations for the implementation of large scale HIV and HCV screening (predictive values)

Predictive value of VIKIA screening test for HIV: In the overall series of 1231 samples, 64 were found positive with the screening test: 55 were confirmed positive, 7 indeterminate and 2 negative giving a PPV of 96.5%.

Comparing the two series, the PPV measured with VIKIA HIV1/2 in young women (i.e., 100%) was significantly higher than that of 87.5% measured in older women ($P < 0.05$). However, when indeterminate results were included in the analysis, the PPV measured with VIKIA test in young women (i.e., 91%) was not different than the PPV of 73.7% found in the older cohort.

Predictive value of screening tests for HCV: Indeterminate results of LIA and/or PCR HCV were not considered for the calculation of predictive values. (1) Positive predictive value of Monolisa HCV test. In the whole series of 1231 samples, 35 were found reproducibly positive: 16 were confirmed seropositive by the LIA-HCV assay, 11 were indeterminate and 8 were confirmed negative, giving a PPV of 66.7% for the screening assay. However, the PPV (88.2%) measured with the Monolisa HCV test in women > 50 years old was significantly higher than that of 14.3% measured in young women ($P < 0.01$); (2) Predictive value of test ratio (TR) results measured with the HCV Monolisa test (Table 3). Using the Monolisa test, when the TR was found to be between 1 and 3 (i.e., TR ≤ 50% TRmax), the rate of positive confirmation was 1/9 with LIA-HCV, and 0/13 with PCR. By contrast, when the TR of the Monolisa test was greater than 3 (TR > 50% TRmax), the probability of positive confirmation was 15/16 with the LIA ($P < 0.01$) and 14/21 with PCR ($P < 0.01$, compared to lesser TR).

Table 3 Predictive values of the Monolisa test ratio in both series of samples

MONOLISA HCV-Ab Ag	Series	N+	LIA-HCV			PPV, %	PCR-RNA-HCV			PPV, %
Test ratio			Neg	Pos	Ind		Neg	Pos	Ind	
(1-3)	1	10	6	0	4	0	6	0	4[1]	0
(> 3)	1	7	1	1	5	50	6	1	0	14.3
(1-3)	2	7	2	1	4	33.3	7	0	0	0
(> 3)	2	14	0	14	0	100	1	13	0	92.9
(1-3)	1 + 2	17	8	1	8	11.1	13	0	4[1]	0
(> 3)	1 + 2	21	1	15	5	93.8	7	14	0	66.7

[1]Four samples [whose test ratio = (1-3)] were not tested with polymerase chain reaction (PCR) because Monolisa results were not reproducible and the Innotest was negative in each case. Ind: Indeterminate result; PPV: Positive predictive value of hepatitis C virus (HCV) Monolisa test for antibody or RNA detection; Series 1: Young women; Series 2: Women over 50 years old; Series (1 + 2): Overall cohort (young and older women); N+: Numbers of positive results with Monolisa HCV according to the test ratio value.

Predictive value of LIA-HCV bands: Out of 17 LIA positive profiles, NS3 and C1 bands were clearly predominant in 16/17 (94.1%); C2 and NS4 were represented in 15/17 (88.2%) and 8/17 (47.1%) respectively, whereas E2 and NS5 were poorly represented in 3/17 samples (17.6%).

The intensity of the most represented band, NS3, was analyzed with regard to its predictive value of a positive PCR result. There was an obvious association between the intensity of the NS3 HCV band and HCV viraemia. This association was highly significant when the NS3 intensity was $\geqslant 3$ ($P < 0.01$). Similarly, there was a significant association between the viraemia of HCV and the coexistence of both C1 and NS3 bands, when the intensity was > 2 ($P < 0.01$). On the contrary, there was no significant association between the intensity of the C1 band and HCV viraemia ($P > 0.05$).

Sample No. 6 (Table 4) had a TR > 5 with two EIA tests (Monolisa HCV and Innotest HCV) and an isolated NS3 band (at 4+ intensity), and therefore was classified as indeterminate according to the manufacturers' recommendations; it was later found to be positive for PCR-HCV-RNA. This result supports the hypothesis that any reactivity in an isolated NS3 band may be indicative of an HCV seroconversion[18].

DISCUSSION

The HIV seroprevalence measured in these populations of young (4.1%) and older (6.1%) women was of the same magnitude. The overall HIV seroprevalence of 4.5% estimated by our study in women is higher than 1.3% that was notified for the general population of Mali[11]. Of note, the rate of HIV infection observed in our study is in the range of prevalences (from 1% to 5%) that was reported in West Africa[9]. It is important to stress that our study concerned exclusively females living mainly in urban areas. According to the EDSM-IV report, the proportion of men and women positive for HIV-2 and whose age varies between 15 and 49 years

Table 4 LIA profiles and polymerase chain reaction results

No.	HCV LIA profile (bands' intensity)						PCR HCV	Viral load (IU/mL)
	C1	C2	E2	NS3	NS4	NS5		
1	4	3	0	4	1	0	pos	32 350
2	3	1	0	4	0	0	pos	1 442 298
3	1	1	0	0.5	0	0	neg	Not detected
4	4	2	0	4	0	0	pos	2 701 467
5	4	3	0	4	0.5	0.5	pos	3 247 624
6	0	0	0	4	0	0	pos	290 103
7	4	2	0	4	3	0	pos	98 440
8	4	4	0	4	0	0	pos	20 503
9	3	2	1	4	0	0	pos	683 959
10	3	1	0	2	0	0	neg	Not detected
11	3	1	0	4	0	2	pos	6 369 090
12	4	2	0	4	1	3	pos	831 422
13	4	3	2	4	0	0	pos	12 352 743
14	3	1	0	4	0.5	0	pos	4 019 864
15	2	3	4	4	4	0	pos	< 12
16	4	1	0	3	2	0	pos	1600
17	1	0	0	0	1	0	neg	Not detected
BP	16	15	3	16	8	3		

C1 and C2: Core protein 1 and 2 for hepatitis C virus (HCV); E2: Transmembrane protein of HCV; NS3, NS4 and NS5: Nonstructural proteins of HCV. PCR: Polymerase chain reaction; BP: Band presence.

is low (0.2%) indicating that HIV-1 predominates over HIV-2[11]. Our study confirms this observation and further indicates that there is significantly more HIV-2 in women > 50 years (5/14) than in young females (2/41), ($P < 0.01$). The HIV prevalence rate of 4.1%, measured in pregnant women in this study is similar to that of 7.9% (18/288) obtained in a population of pregnant women, in the neighboring country of Burkina Faso[19]. Regarding the predictive positive values measured with VIKIA HIV-1/2, our study shows that this test is more efficient in Malian young women compared to older ones (100% *vs* 85.7%; $P < 0.05$). The rate of false positive HIV results (VIKIA pos; LIA neg) in the two subpopulations, (0% in young women *vs* 10.5% in older ones) may explain this difference.

Our study is the first to determine molecular HCV epidemiology and to evaluate the performance of EIA HCV tests in Mali. HCV seroprevalence was 3.3% among Malian blood donors[7]. That frequency is lower than that of 6.5% measured in our study in women over 50 years, but remains well above that of 0.2% measured in young women.

The HCV prevalence rates of 0.2% and 6.5% measured respectively in young and older women in our study are in the range of prevalences (0.1%-13.8%) documented in sub-Saharan Africa[6]. Similarly, the overall prevalence of HCV (1.3%) measured in the present study in women is in the range of prevalence (1.1%-5.5%) reported in six West African countries[6]. That prevalence could be extrapolated to the general Malian population. However, the population of our study is essentially urban. It would be interesting to conduct an additional study in rural areas of Mali to confirm the quality of our data.

The prevalence of 0.2% HCV as measured by our

study in pregnant women is of the same order of magnitude than the prevalences of 0.14% and 0.18% respectively reported in previous studies[16,20]. In our study, only one pregnant woman (AD3) of two HCV seropositives was found PCR positive (HCV-RNA+) and infected with genotype 2. In the other subject, OM4, the sample exhibited a discrepancy with the two tests (Innotest pos/Monolisa neg). This woman, OM4, tested negative twice by PCR on two separate samples collected at an interval of 11 mo. This suggests either a very old HCV infection with virtual disappearance of specific antibodies, or false positive results of EIA and LIA due to aspecific antibodies. In older women HCV seroprevalence is 6.5%, therefore significantly higher than in younger females (0.2%, $P < 0.01$). The significant variation of anti-HCV prevalence according to the age of women (> 50 years *vs* younger), 6.5% *vs* 0.2%, may suggest the hypothesis that exposure of women > 50 years to HCV infection is usually an old one. Accordingly, the prevalence of HCV-RNA was 5.6% *vs* 0.1 % in the two groups.

The proportion of HCV positive women was significantly higher in rural areas (6/91) compared to urban residence (11/1125, $P < 0.01$). That would also explain the discrepancy between the HCV seroprevalence of 0.2% in young women of whom only 2.63% came from rural areas and 6.5% observed in older women of whom 29.13% came from rural areas. The distribution of HCV genotypes 1 and 2 revealed in Mali by our study is similar to that reported in West Africa[21-23]. Our study reveals the predominance of HCV genotype 2 (11/13 or 84.6%) known to be easier to treat than genotype 1. HCV prevalence measured in our study in young women (0.2%) is close to data obtained in pregnant women in two countries of West Africa namely Niger (0%) and Benin (0.7%)[24].

As far as the performance of third-generation HCV EIA tests in the African region are concerned, there are quite controversial opinions in the literature. Indeed, some studies report poor performance of EIA tests for third and even the fourth generation[20,21,25-28]. Conversely, others have observed good performance using these tests[29-38]. In the present study, our data indicate the poor performance of EIA tests both in terms of predictive positive value (14.3% *vs* 7.4%) and in terms of specificity (42.9% *vs* 55.6) for false positive results obtained using both Monolisa (mixed test Ag-Ab) and Innotest (Ab test only), in the population of pregnant women where the prevalence of HCV is particularly low. Of note, the positive predictive values as well as the rates of false positive reactions were not significantly different using either of the two tests.

On the other hand, our observations confirm the better performance of Monolisa test used as a screening test (with 2/21 or 9.5% false positive; PPV at 88.2%) in the population of women over 50 years where the prevalence was higher.

The rates of false positive samples measured with the Monolisa test in the two populations (42.9% *vs* 9.5%) were significantly different ($P < 0.05$). Equally the positive predictive values of the Monolisa test were significantly different in the 2 populations (14.3% *vs* 88.2%; $P < 0.01$).

We also observed EIA false positive reactions which could be related to a cross-reaction by antibodies caused by poly-immunization against bacterias or parasites[39,40].

As far as the tests performance are concerned, our results suggest that for TR values > 3 (i.e., TR > 50% TRmax), the repetition of the EIA test and confirmation by the INNO-LIA HCV test are not required to anticipate a confirmed HCV infection (PPV at 93.8%; $P < 0.01$) and positive viraemia (PPV at 66.7%; $P < 0.01$).

Taking into account the 2 subpopulations separately, when TR is in the range of 1 to 3 (i.e., TR ⩽ 50% TRmax), there is not a significant difference between the PPV of TR measured with LIA HCV (0% *vs* 33.33%) or PCR (0% *vs* 0%) in populations of young and older women. In contrast, when TR > 50% TRmax, the PPV of test ratio measured in older women with LIA HCV (100%) is significantly higher than that measured in younger women (50%, $P < 0.01$). According to PCR analysis, the PPV of TR measured in older women (92.9%) is significantly higher than 14.3% calculated in pregnant women ($P < 0.01$). This supports the recommendation that the patient's age should be taken into account in the interpretation algorithm.

Our data confirm the findings of other authors who reported a high degree of accuracy between anti-HCV TR and HCV viraemia[38,41].

In all samples analyzed, no HIV/HCV co-infection was detected but in each population we observed one case of HIV/HCV co-indetermination, with the following LIA profiles: p17 (2+)/C1 (1+) and gp41 (2+)/NS3 (0.5+).

In summary, we have observed a high prevalence of HIV infection in both subpopulations of pregnant women and women of more than 50 years old. We have also reported a high prevalence of HCV 6.5% in older women. Our data indicate a good efficiency of VIKIA HIV 1/2 (BioMérieux) in pregnant women and a high performance of Monolisa HCV Ag/Ab Ultra (Biorad) in older women. In addition we have proposed an interpretation algorithm for the clinical diagnosis of HCV infection in Mali. This algorithm could be useful in the West African population. Furthermore we have reported the importance of INNO-LIA HCV test in an African population since it may detect an earlier HCV seroconversion.

Our data stress the importance of both infections in Mali, and the need to organize the management of viral hepatitis in general in Mali together with the endemic HBV infection at a prevalence of 14.7% based on HBsAg assays[42].

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COMMENTS

Background

In Sub-Saharan Africa, the prevalence of hepatitis C virus (HCV) varies between 0.1% and 13.8 %. In Mali, HCV seroprevalence is estimated at 3.3% in the population of Malian blood donors, but is not known accurately in the population of pregnant women, and even less in the general population. Furthermore the molecular epidemiology of HCV is unknown. As for human immunodeficiency virus (HIV), the seroprevalence is 1.3% in the general population. To prevent HIV vertical transmission, the screening of infection in pregnant women is undertaken with few rapid tests in health centers providing antenatal care. The VIKIA HIV 1/2 3rd generation test (BioMerieux) is known to be highly sensitive and specific, and does not require complex instrumentation. It is therefore well suited to facilitate HIV testing, especially in poorer areas where technical facilities are unavailable.

Research frontiers

As for the performance of the third-generation HCV enzyme immuno-assay (EIA) tests (EIA3) in the African region, there are quite controversial reports in published studies. In the present work, the authors demonstrate that the specificity is high in women over 50 years old, but much lower in the pregnant women cohort. Indeed, when test ratio (TR) > 50% TRmax, the probability for EIA3 HCV test to give a confirmed antibody reaction is 2 times higher in older women than in younger ones. The probability of a confirmed positive polymerase chain reaction (PCR) reaction (i.e., to detect HCV-RNA) is 6 times higher in older women than in younger ones.

Innovations and breakthroughs

To reduce HCV-EIA false positive results, the World Health Organization as well as other authors recommend replicating tests by using two different serological assays. In the present study, the authors propose a new interpretation algorithm which balances effectiveness and cost, based on the signal/cut-off ratio, and applicable in Mali to the general population. This algorithm may be generalized for use in other West African countries. As for the interpretation of INNO-LIA HCV assays, a single reactive NS3 band may be indicative of seroconversion. Such samples will be classified as indeterminate as per manufacturer's instructions. In this case, it is recommended to test a sample from the same patient drawn a few weeks later. In this study, the authors demonstrated that the intensity of single NS3 bands higher than 3 may be indicative of viraemia. Indeed, in one sample of our study with an isolated NS3 band at 4+ intensity, the patient was later found positive by PCR analysis, thus indicating earlier seroconversion and active HCV infection.

Applications

The overall prevalence of HCV (1.3%) measured in our study in women is within the range of data (from 1.1% to 5.5%) collected in six West African countries. The 1.3% prevalence may be extrapolated to the Malian general population. However, since our population under study was essentially urban, an additional cohort in rural areas of Mali would be useful to confirm the estimations.

Terminology

EIA3: The detection of anti-HCV antibodies in plasma or serum is based on the use of third-generation EIA, that detects mixtures of antibodies directed against various HCV epitopes. Recombinant antigens are used to capture circulating anti-HCV antibodies onto the wells of microtiter plates, microbeads, or specific holders adapted to closed automated devices. The presence of anti-HCV antibodies is revealed by anti-antibodies labeled with an enzyme that catalyzes the transformation of a substrate into a colored compound. The optical density (OD) ratio of the reaction (sample OD/internal control OD) is proportional to the amount of antibodies in the serum or plasma sample; Predictive values: The positive predictive value is the probability that when the test is reactive, the specimen does contain antibody to HCV. This may be calculated by using the simple formula: true positives/(true positives + false positives) which will give an approximate value; INNO-LIA™ HCV score: Based on the principle of an enzyme immunoassay. A sample is incubated in a compartment with a strip. The anti-HCV antibodies possibly present in the sample will bind to the HCV antigen bands fixed to the strip. Then conjugated goat anti-human immunoglobulin G (H + L) coupled to alkaline phosphatase is added and binds to the antigen/antibody complex formed previously. Incubation with enzyme substrate produces a brown color whose intensity is proportional to the amount of specific antibodies to HCV-related antigen bands. The color development is stopped by adding sulfuric acid.

Peer review

The manuscript is interesting, well done and well written.

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ORIGINAL ARTICLE

Ultrasonogram of hepatocellular carcinoma is associated with outcome after radiofrequency ablation

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Abstract

AIM: To investigate the association between B-mode ultrasound classification of small hepatocellular carcinoma (HCC) and outcome after radiofrequency ablation (RFA).

METHODS: Ninety-seven cases of HCC treated using RFA between April 2001 and March 2006 were reviewed. Ultrasound images were classified as follows: type 1, with halo (n = 29); and type 2, without halo (n = 68). Type 2 was further categorized into three subgroups: type 2a, homogenous hyperechoic (n = 9); type 2b, hypoechoic with smooth margins (n = 43); and type 2c (n = 16), hypoechoic with irregular or unclear margins. Patients with type 2a HCC were excluded from analysis due to the small number of cases.

RESULTS: Two year recurrence rates for type 2b, type 1 and type 2c were 26%, 42% and 69%, respectively, with significant differences between type 2b and type 2c (P < 0.01), and between type 1 and type 2c (P < 0.05). Five year survival rates were 89%, 43% and 65%, respectively. Survival was significantly longer for type 2b than for other types (type 1 *vs* type 2b, P < 0.01; type 2b *vs* type 2c, P < 0.05). On univariate analysis, factors contributing to recurrence were number of tumors, tumor stage, serum level of lens culinaris agglutinin-reactive alpha-fetoprotein and ultrasound classification (P < 0.05). Factors contributing to survival were tumor stage and ultrasound classification (P < 0.05). Multivariate analysis identified ultrasound classification as the only factor independently associated with both recurrence and survival (P < 0.05).

CONCLUSION: B-mode ultrasound classification of small HCC is a predictive factor for outcome after RFA.

Key words: B-mode ultrasound; Hepatocellular carcinoma; Radiofrequency ablation; Recurrence; Prognosis

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INTRODUCTION

Treatment strategies for hepatocellular carcinoma (HCC) are decided on the basis of tumor size and number, liver function and performance status[1,2]. Percutaneous local treatments that are less invasive than resection are performed for small HCCs that are unsuitable for resection, with the indications of ≤ 3 lesions, each with diameter ≤ 3 cm, in accordance with the Japanese guidelines[2] and the practice guidelines of the American Association for the Study of Liver Diseases[1].

Percutaneous radiofrequency ablation (RFA) is a well established local treatment for unresectable small HCC[3,4]. RFA is a curative treatment and achieves not only superior local control of the disease, but also better prognosis compared to percutaneous ethanol injection therapy (PEIT)[5,6]. Accordingly, RFA is now recommended over PEIT for the treatment of small HCC. Recently, RFA has also been adopted for patients with resectable early HCC, defined as single tumors > 2 cm in diameter or up to 3 nodules < 3 cm in diameter, with performance status 0 and Child-Pugh class A or B[7].

However, rapid aggressive recurrence with vascular invasion[8-10], intrahepatic dissemination[11,12], seeding or metastasis[13,14] has been reported after RFA. In particular, the risk of seeding is high in patients with poorly differentiated HCC[15]. Furthermore, the prognosis following RFA for poorly differentiated HCC is reportedly unfavorable[16,17]. A large proportion of patients with poorly differentiated HCC show microscopic vascular invasion and intrahepatic metastasis, even when the tumor is small[18]. As a result, curative treatment cannot be achieved using RFA alone and the procedure may thus cause dissemination or metastasis. Clinical diagnosis of poorly differentiated HCC with high-grade malignancy is therefore crucial when determining treatment strategies for small HCC.

Small HCCs show various images on B-mode ultrasound. However, the correlation between B-mode ultrasound image and prognosis has not been elucidated. We have previously reported that classification on B-mode ultrasonography of small hypervascular HCC correlated with histological differentiation and serum level of lens culinaris agglutinin-reactive alpha-feto protein (AFP-L3), an indicator of poor prognosis[19]. In particular, the presence of irregular or unclear margins was very important in screening for small, poorly differentiated HCC. The aim of this study was to determine whether B-mode ultrasound classification is associated with recurrence and survival after RFA.

MATERIALS AND METHODS

Patients

Our prospective database of 97 patients with initial hypervascular HCC (≤ 3 tumors, all ≤ 3 cm in diameter) who had undergone RFA between April 2001 and March 2006 was reviewed. Diagnosis of hypervascular HCC was based on the findings of tumor staining during the arterial phase of contrast-enhanced computed tomography (CT), dynamic magnetic resonance imaging (MRI) or contrast ultrasonography. If any of these diagnostic imaging techniques showed tumor stain in the arterial phase that was washed out in the equilibrium phase, imaging diagnosis was considered definitive. In all patients, tumor stage (tumor-node-metastasis classification as described by the Liver Cancer Study of Japan), etiology of hepatitis, Child-Pugh classification, levels of tumor markers (AFP, AFP-L3 and des-gamma-carboxy prothrombin), fibrosis stage and activity grade of the biopsied liver tissue using the new Inuyama classification[20] were evaluated before RFA. Eligibility criteria for RFA were as follows: (1) no vascular invasion on imaging diagnosis; (2) no severe ascites; (3) platelet count ≥ $5 \times 10^4/mm^3$; (4) prothrombin time ≥ 50%; (5) total bilirubin < 3 mg/dL; (6) no distant metastases; and (7) in principle, ≤ 3 tumors, all ≤ 3 cm in diameter. No exclusion criteria were set in terms of tumor location (i.e., near main vessels, adjacent organs). Furthermore, all patients with recurrent HCC underwent iterative RFA even when the above criteria for tumor size and number were not met, as long as complete ablation was considered achievable. Written informed consent was obtained from each enrolled patient and the protocol was approved by our institutional review board.

RFA technique

Percutaneous RFA using the Cool-tip RF system (Valleylab, Boulder, CO, United States) was performed under ultrasound guidance in all patients. Artificial pleural effusion or artificial ascites was produced using saline when necessary[21]. The impedance control mode was used with a 17-gauge, cooled-tip electrode with a 2 or 3 cm exposed tip. Ablation was started at 40 W for the 2 cm exposed tip and 60 W for the 3 cm exposed tip. Power was increased at a rate of 10 W/min. When a rapid increase in impedance occurred, output was automatically stopped and ablation was restarted after a short time at an output 10 W lower. Duration of a single ablation was 6 min for the 2 cm electrode and 12 min for the 3 cm electrode. After RF exposure, temperature of the needle tip was measured. When the temperature was below 65 ℃, additional ablation was performed. The electrode track was not treated by thermo-coagulation in any patients, to prevent seeding or hemorrhage.

Assessment of response and follow-up

Treatment response was assessed by contrast-enhanced CT or MRI at 1-3 d after the final session. Complete response was defined as no enhancement in the entire lesion with a safety margin on imaging. Additional ablation was performed until complete ablation was confirmed in each nodule. All patients were followed up on an outpatient basis every 3-4 mo using contrast-enhanced CT or MRI and measurement of tumor marker levels.

B-mode ultrasound imaging

We used either a SONOLINE Elegra™ Ultrasound Platform (Siemens Medical Systems, Erlangen, Germany)

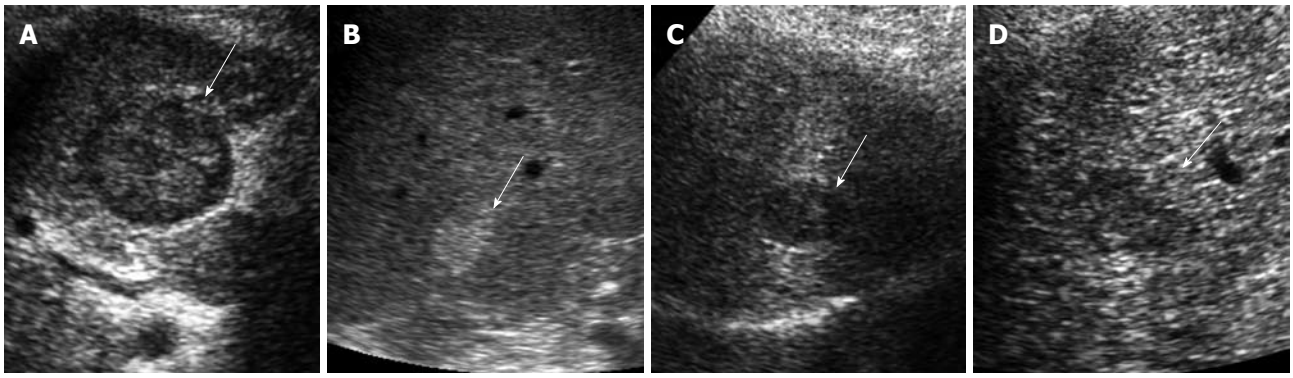

Figure 1 Classification of B-mode ultrasonographic images of small hepatocellular carcinoma. A-D: Hepatocellular carcinoma nodules < 3 cm in diameter were classified into two groups using B-mode ultrasonography: Type 1 with halo (A) and type 2 without halo. Type 2 was then further categorized into three subgroups: Type 2a, homogenous hyperechoic (B); Type 2b, hypoechoic with smooth margins (C); Type 2c, hypoechoic with irregular or unclear margins (D). Hepatocellular carcinoma nodules are indicated by arrows.

Table 1 Comparison of patient characteristics according to B-mode ultrasound-based classification

	Type 1 (*n* = 29)	Type 2b (*n* = 43)	Type 2c (*n* = 16)	*P* value
Age (yr)	66.4 ± 9.5	66.9 ± 8.7	69.3 ± 6.8	0.554
Gender (male/female)	23/6	25/18	8/8	0.085
HCV (positive/negative)	28/1	36/7	14/2	0.241
Number of tumors	1.2 ± 0.4	1.2 ± 0.5	1.6 ± 0.7	0.046
Size of tumor (mm)	22.5 ± 3.8	19.0 ± 5.2	23.1 ± 5.6	0.003
Child-Pugh classification (A/B)	16/13	31/12	9/7	0.272
Tumor stage (Ⅰ/Ⅱ/Ⅲ)	10/15/4	28/13/2	4/7/5	0.006
Activity grade (A0, 1/2, 3)	7/22	14/29	10/6	0.032
Fibrosis stage (F0-2/3, 4)	8/21	10/33	7/9	0.298
AFP (ng/mL)	124 ± 246	118 ± 274	207 ± 406	0.564
AFP-L3 (%)	7.3 ± 17.0	5.8 ± 16.5	17.5 ± 25.5	0.098
DCP (mAU/mL)	223 ± 489	210 ± 482	299 ± 486	0.817

Data are presented as mean ± SD or *n*/*N*. HCV: Hepatitis C virus; AFP: Alpha-fetoprotein; AFP-L3: Lens culinaris agglutinin-reactive alpha-fetoprotein; DCP: Des-gamma-carboxy prothrombin.

with a 3.5C40 convex probe or a SSA-770A ultrasound system (Toshiba Medical Systems, Tochigi, Japan) with a PVT-674BT ultrasound probe. Tissue harmonic imaging was performed in B-mode.

B-mode ultrasound classification

The B-mode ultrasound classification of small HCC we reported previously was used[19]. Nodules with a halo were regarded as type 1 and halo-free nodules were regarded as type 2. In addition, type 2 nodules were further classified based on the internal echo level and marginal features; uniform hyperechoic nodules were evaluated as type 2a, hypoechoic nodules with regular margins as type 2b and hypoechoic nodules with irregular or unclear margins as type 2c. B-mode ultrasound images were obtained within 1 mo before RFA. All recorded ultrasound images were analyzed by two skilled hepatologists (10 and 19 years of experience in abdominal ultrasonography) who were blinded to patient names. When a discrepancy existed in interpretation between the two hepatologists, a consensus was reached through discussion. If HCCs comprised two or three nodules, the largest nodule was selected and classified using our B-mode classification. B-mode ultrasound classified 29 cases as type 1, 9 as type 2a, 43 as type 2b, and 16 as type 2c. Given the small number of patients with type 2a HCC, these cases were excluded from analysis (Figure 1).

Statistical analysis

One factor analysis of variance and the Scheffe test were used to analyze continuous variables. Fisher's exact test or the χ^2 test were used to analyze categorical variables. Cumulative recurrence-free survival rates and cumulative survival rates according to B-mode ultrasound classification were constructed using Kaplan-Meier methods and compared using the log-rank test. Uni- and multivariate analyses using a Cox proportional hazard regression model were performed for factors contributing to tumor recurrence and survival. Results were expressed as hazard ratio with 95%CI. $P < 0.05$ was considered statistically significant for all analyses using SPSS Statistics Version 19 software (IBM, Tokyo, Japan).

RESULTS

The median follow-up interval was 1018 d. Two year recurrence rates for type 2b, type 1 and type 2c were 26%, 42% and 69%, respectively. Significant differences were seen between type 2b and type 2c ($P < 0.01$), and between type 1 and type 2c ($P < 0.05$). Five year survival rates were 89%, 43% and 65%, respectively. Survival was significantly longer for type 2b than for other groups (type 1 *vs* type 2b, $P < 0.01$; type 2b *vs* type 2c, $P < 0.05$).

Patient background variables at baseline according to B-mode ultrasound classification are compared in Table 1. Significant differences were evident among groups in terms of number of tumors, tumor size, tumor stage and activity grade of hepatitis. Mean tumor size was smaller in type 2b than in other types. Mean number of tumors was smaller in type 2b than in type 2c. High tumor stage was more frequent in type 2c than in other types. Severe activity grade of hepatitis was likewise more frequent

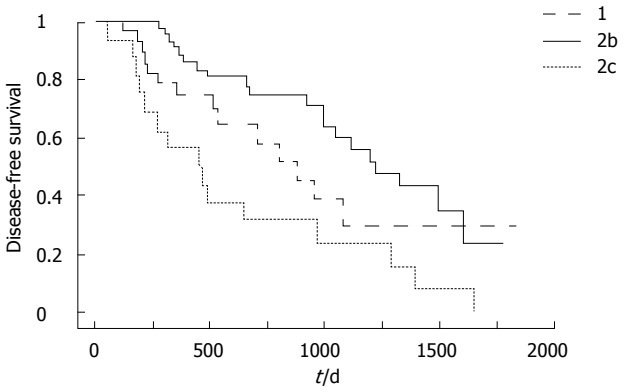

Figure 2 Recurrence-free survival curves according to B-mode ultrasound classification. Recurrence-free survival was significantly shorter for type 2c hepatocellular carcinoma than for other types. $P = 0.0454$ type 1 *vs* type 2c; $P = 0.0005$ type 2b *vs* type 2c.

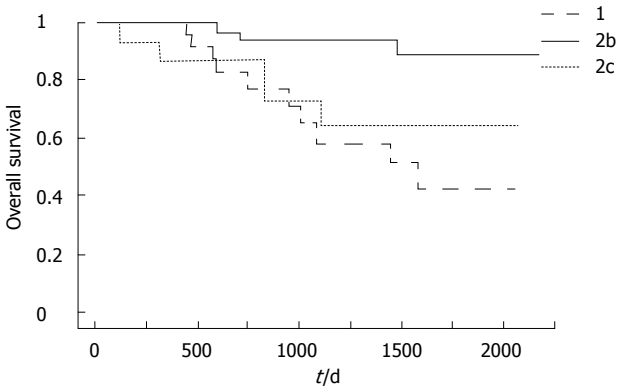

Figure 3 Survival curves according to B-mode ultrasound classification. Survival was significantly longer for type 2b than for other types. $P = 0.0006$ type 1 *vs* type 2b; $P = 0.0165$ type 2b *vs* type 2c; $P = 0.4473$ type 1 *vs* type 2c.

in type 2c than in other types. Mean AFP-L3 level was higher in type 2c than in other types.

Recurrence-free survival curves according to B-mode ultrasound classification are compared in Figure 2. Recurrence-free survival was significantly shorter for type 2c HCC than for other types.

Survival curves according to B-mode ultrasound classification are compared in Figure 3. Survival was significantly longer for type 2b than for other groups. No significant difference in survival was evident between types 1 and 2c.

Results of univariate analysis of background variables associated with tumor recurrence are shown in Table 2. Number of tumors, tumor stage, AFP-L3 levels and B-mode ultrasound classification were identified as significant contributing factors for recurrence after RFA. These significant variables were then entered into multivariate analysis. The results of multivariate analysis are shown in Table 3, with type 2c of B-mode ultrasound classification identified as the only independent factor contributing to tumor recurrence.

The results of univariate analysis of background variables associated with survival are shown in Table 4. Tumor stage and B-mode ultrasound classification were identified as significant contributing factors for survival. All significant variables on univariate analysis were then entered into multivariate analysis. The results of multivariate analysis are shown in Table 5, with type 1 of B-mode ultrasound classification identified as the only independent factor contributing to survival.

DISCUSSION

This review of a prospective database for patients who had undergone RFA for primary HCC found B-mode ultrasound image classification as an independent factor strongly influencing post-RFA recurrence and disease outcomes. This result supports the earlier finding that the B-mode ultrasound image classification we devised is capable of evaluating the malignant potential of HCC[19].

The most common B-mode ultrasound image classification for primary HCCs ⩽ 3 cm in diameter that had undergone RFA was type 2b, followed by types 1b, 2c and 2a, in that order. Substantial bias in the distribution of ultrasound types was thus evident. We have previously reported that type 2a HCC shows the smallest mean diameter in B-mode ultrasound classification and represents well-differentiated HCC with fat deposition[19]. Type 2a can therefore be considered as the ultrasound classification associated with the lowest malignant potential. Our present database of patients with hypervascular HCC included only 9 type 2a patients and data for these cases were excluded from analyses due to insufficient numbers. However, type 2a HCC seems likely to represent the patient group associated with the best prognosis.

Type 2b showed a longer interval until recurrence and better outcomes compared with types 1 and 2c. Of course, mean tumor diameter is smaller in type 2b than in types 1 and 2c and tumor stage also shows a higher proportion of early-stage cases. Outcomes for type 2b could thus be due to these reasons. However, multivariate analysis identified ultrasound categorization as an independent factor exerting greater influence on recurrence and survival than either tumor diameter or stage. For that reason, the malignant potential of type 2b HCC can be considered lower than that of types 1 and 2c.

Type 1 in the B-mode ultrasound image classification represents HCC with a clear margin accompanied by a halo. The halo represents a fibrous capsule, a finding commonly seen in dedifferentiated and moderately differentiated HCCs during the course of multistep carcinogenesis. In contrast, a type 2c ultrasound image is commonly seen in poorly differentiated HCCs, showing no halo and irregular or unclear margins[19]. In addition, a type 2c ultrasound image is frequently seen in HCCs that are positive for AFP-L3 and type 2c HCCs thus have higher malignant potential than type 1 HCCs[19]. When the recurrence-free curve following RFA was stratified for types 2b, 1 and 2c, type 2c was seen to include the most cases of early recurrence. This difference can be thought to reflect the malignant potential of HCC. Conversely, we found no difference in survival rates between

Table 2 Univariate analysis of factors contributing to recurrence

Variables	HR	95%CI	*P* value
Age (yr)	1.007	0.972-1.043	0.717
Gender			
Female	1		
Male	1.036	0.577-1.859	0.906
HCV			
Negative	1		
Positive	1.176	0.527-2.625	0.693
Number of tumors	1.557	1.009-2.404	0.045
Size of tumor (mm)	1.035	0.980-1.092	0.214
Child-Pugh classification			
A	1		
B	1.481	0.838-2.617	0.177
Tumor stage			
Ⅰ	1		
Ⅱ	1.214	0.657-2.244	0.535
Ⅲ	5.055	2.123-12.034	0.000
Activity grade			
A0, 1	1		
A2, 3	1.346	0.745-2.430	0.325
Fibrosis stage			
F0-2	1		
F3, 4	1.323	0.689-2.540	0.401
AFP (ng/mL)	1.000	1.000-1.001	0.318
AFP-L3 (%)	1.017	1.004-1.030	0.023
DCP (mAU/mL)	1.000	1.000-1.001	0.332
B-mode classification			
Type 2b	1		
Type 1	1.531	0.767-3.053	0.227
Type 2c	3.179	1.623-6.227	0.001

HR: Hazard ratio; HCV: Hepatitis C virus; AFP: Alpha-fetoprotein; AFP-L3: Lens culinaris agglutinin-reactive alpha-fetoprotein; DCP: Des-gamma-carboxy prothrombin.

Table 3 Multivariate analysis of factors contributing to recurrence

Variables	HR	95%CI	*P* value
n	1.411	0.719-2.770	0.317
Tumor stage			
Ⅰ	1		
Ⅱ	0.645	0.283-1.467	0.296
Ⅲ	2.540	0.784-8.224	0.120
AFP-L3			
Negative	1		
Positive	1.531	0.642-3.649	0.337
B-mode classification			
Type 2b	1		
Type 1	1.850	0.878-3.899	0.106
Type 2c	2.438	1.107-5.373	0.027

HR: Hazard ratio; AFP-L3: Lens culinaris agglutinin-reactive alpha-fetoprotein.

types 1 and 2c. HCC does not just show a high recurrence rate; it is a cancer that also undergoes repeated recurrence, as a result of which the level of compliance with the follow-up schedule and administration of treatment for recurrent lesions also greatly influence the outcome. The patient database used in the present study was prospective, in accordance with the predetermined

Table 4 Univariate analysis of factors contributing to survival

Variables	HR	95%CI	*P* value
Age (yr)	0.979	0.929-1.032	0.440
Gender			
Female	1		
Male	1.161	0.435-3.096	0.766
HCV			
Negative	1		
Positive	0.947	0.218-4.124	0.943
Number of tumors	1.700	0.840-3.440	0.140
Size of tumor (mm)	1.030	0.945-1.123	0.504
Child-Pugh classification			
A	1		
B	1.789	0.710-4.512	0.218
Tumor stage			
Ⅰ	1		
Ⅱ	1.588	0.551-4.579	0.392
Ⅲ	3.847	1.081-13.690	0.037
Activity grade			
A0, 1	1		
A2, 3	1.978	0.698-5.606	0.200
Fibrosis stage			
F0-2	1		
F3, 4	1.292	0.425-3.930	0.652
AFP (ng/mL)	1.001	1.000-1.002	0.087
AFP-L3 (%)	1.012	0.995-1.031	0.212
DCP (mAU/mL)	1.000	0.999-1.001	0.919
B-mode classification			
Type 2b	1		
Type 1	6.911	1.897-25.176	0.003
Type 2c	4.466	1.066-18.709	0.041

HR: Hazard ratio; HCV: Hepatitis C virus; AFP: Alpha-fetoprotein; AFP-L3: Lens culinaris agglutinin-reactive alpha-fetoprotein; DCP: Des-gamma-carboxy prothrombin.

Table 5 Multivariate analysis of factors contributing to survival

Variables	HR	95%CI	*P* value
Tumor stage			
Ⅰ	1		
Ⅱ	1.189	0.406-3.481	0.752
Ⅲ	3.763	0.896-15.796	0.070
B-mode classification			
Type 2b	1		
Type 1	7.146	1.924-26.538	0.003
Type 2c	3.055	0.668-13.970	0.150

HR: Hazard ratio.

follow-up schedule. For that reason we think that compliance with the follow-up schedule exerted little effect on outcomes in this study. However, the possibility that treatment at the time of recurrence influenced the results cannot be ignored. We actively performed RFA for our patients, not only in cases where they satisfied the indications for RFA at the time of recurrence, but even when criteria of tumor size and number were not met, as long as the imaging findings were evaluated as suggesting that the lesions could be controlled. Radical retreatment rates, including iterative RFA or resection for

type 1, type 2b and type 2c, were 93%, 95% and 80%, respectively. No significant differences were seen among radical re-treatment rates according to ultrasound classification. Our analysis of prognostic factors in this study did not include the treatment method at the time of recurrence. However, early detection of recurrence due to the prudent follow-up schedule and the effectiveness of our active treatment of recurrent lesions may have been reasons for the lack of significant differences in survival rate between types 1 and 2c.

Studies to date have identified various risk factors for recurrence following RFA for HCC, including tumor diameter[22-27], tumor number[28-30], tumor stage[24], histological poor differentiation[24], insufficient safety margin[25,29,31], a tumor location[25,27] that is problematic for RFA, such as adjacent to a large blood vessel or the liver surface, hepatitis C virus[28] and/or hepatitis B virus infection[23], AFP level[22,25,28], liver fibrosis and platelet count[23,30]. Santambrogio *et al*[32] recently reported that intraoperative ultrasound score can predict recurrent HCC after radical treatment. However, no previous reports have identified B-mode ultrasonogram for small HCC using external ultrasound as a risk factor for recurrence. The present study identified that risk factors for recurrence included, not only the previously reported tumor number, tumor stage and tumor markers, but also ultrasound image type. Moreover, multivariate analysis identified ultrasound image type 2c as the only significant independent risk factor. Ultrasound image type thus seems more closely associated with recurrence of HCC after RFA than previously reported risk factors.

On the other hand, the prognostic factors for RFA that have been reported to date are similar to the risk factors for recurrence; in addition to factors such as tumor diameter, tumor number, tumor stage, tumor differentiation grade and tumor markers, patient age and hepatic function (Child's classification) have also been cited[33-37]. The present study found that tumor stage and B-mode ultrasound image type all represented significant prognostic factors and multivariate analysis identified ultrasound image type as the only significant independent risk factor. Accordingly, B-mode ultrasound image type appears more closely associated with the outcome of RFA than the previously reported risk factors for both recurrence and prognosis. When deciding therapeutic strategies for small HCC in the future, the greatest attention and importance should be placed on the B-mode ultrasound image type.

Ultrasound achieves superior spatial resolution compared with CT and MRI and is the most capable modality for depicting the morphological details of tumors. For that reason, ultrasound is considered to closely reflect the gross morphology of tumors. The gross morphology of HCC is a prognostic factor. With regard to the nodular type, the contiguous multi-nodular type and the single nodular type with extra-nodular growth are reportedly less histologically differentiated than the single nodular type and, because they show higher incidences of vascular invasion and intrahepatic metastasis, recurrence following resection is an early indicator of poor prognosis[38-40]. We think that the reason ultrasound image type is strongly associated with recurrence and outcomes following RFA is that the B-mode ultrasound classification we devised closely reflects the macroscopic type of HCC and enables identification of small HCC with poorer differentiation and higher malignant potential.

Some limitations of the present study were the design as a small-scale retrospective study, with a large degree of bias in the distribution of B-mode ultrasound image types. This prevented us from analyzing post-RFA recurrence and prognosis in relation to type 2a small HCC. We were also unable to investigate the effects of therapy on hepatitis, which presumably influences the recurrence and outcomes of small HCC, or the effects of treatments administered at the time of recurrence. However, even with those limitations, we were able to generate very interesting results indicating that the B-mode ultrasound image type is strongly associated with outcomes following RFA.

In conclusion, this study demonstrated B-mode ultrasound image classification type as a factor that strongly influences outcomes following RFA of small HCC. This new knowledge is likely to influence the therapeutic strategies of physicians treating patients with small HCC. That is, even if a patient satisfies the indications for RFA, the malignant potential of lesions should be evaluated on the basis of B-mode ultrasound image classification and tumor marker levels. Since type 1 and type 2c small HCC have high malignant potential, potentially more effective therapeutic strategies that include other treatment methods, such as resection or concurrent transcatheter arterial chemoembolization *etc*., should be carefully devised rather than simply selecting RFA by default.

COMMENTS

Background

Percutaneous radiofrequency ablation (RFA) is a minor invasive and radical treatment for small hepatocellular carcinomas (HCCs). However, rapid aggressive recurrence with vascular invasion, intrahepatic dissemination, seeding or metastasis has been reported after RFA. The risk of seeding is high in patients with poorly differentiated HCC. Furthermore, the prognosis following RFA for poorly differentiated HCC is reportedly unfavorable. Clinical diagnosis of poorly differentiated HCC with high-grade malignancy is therefore very important when selecting treatment for small HCC.

Research frontiers

Small HCCs show various images on B-mode ultrasound. However, the correlation between B-mode ultrasound image and prognosis has not been elucidated. In the present study, the authors investigated the association between B-mode ultrasound image of small hypervascular HCC and outcome after RFA. The authors have previously reported that classification on B-mode ultrasonography of small hypervascular HCC correlated with histological differentiation.

Innovations and breakthroughs

This is the first study to report that B-mode ultrasound image of small hypervascular HCC is a predictive factor for outcome after RFA. Nodules with a halo and halo-free hypoechoic nodules with irregular or unclear margins have higher malignant potential and treatment for such lesions should be selected with care.

Applications

B-mode ultrasound image of small HCC is likely to influence the therapeutic strategies of physicians treating patients with small HCC.

Terminology

The halo sign is a hypoechoic band around the tumor, corresponding to a thin fibrous capsule around the HCC.

Peer review

The authors report a series of 97 patients affected by HCC undergoing RFA. They demonstrated that the ultrasonographic pattern of HCC predicts the risk of recurrence after RFA. It is an interesting and innovative issue.

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BRIEF ARTICLE

Surgically induced weight loss by gastric bypass improves non alcoholic fatty liver disease in morbid obese patients

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Abstract

AIM: To evaluate the effects of surgical weight loss (Roux-en-Y gastric bypass with a modified Fobi-Capella technique) on non alcoholic fatty liver disease in obese patients.

METHODS: A group of 26 morbidly obese patients aged 45 ± 2 years and with a body mass index > 40 kg/m^2 who underwent open surgical weight loss operations had paired liver biopsies, the first at surgery and the second after 16 ± 3 mo of weight loss. Biopsies were evaluated and compared in a blinded fashion. The presence of metabolic syndrome, anthropometric and biochemical variables were also assessed at baseline and at the time of the second biopsy.

RESULTS: Percentage of excess weight loss was 72.1% ± 6.6%. There was a reduction in prevalence of metabolic syndrome from 57.7% (15 patients) to 7.7% (2 patients) ($P < 0.001$). Any significance difference was observed in aspartate aminotransferase or alanine aminotransferase between pre and postsurgery. There were improvements in steatosis ($P < 0.001$), lobular ($P < 0.001$) and portal ($P < 0.05$) inflammation and fibrosis ($P < 0.001$) at the second biopsy. There were 25 (96.1%) patients with non alcoholic steatohepatitis (NASH) in their index biopsy and only four (15.3%) of the repeat biopsies fulfilled the criteria for NASH. The persistence of fibrosis (F > 1) was present in five patients at second biopsy. Steatosis and fibrosis at surgery were predictors of significant fibrosis postsurgery.

CONCLUSION: Restrictive mildly malabsorptive surgery provides significant weight loss, resolution of metabolic syndrome and associated abnormal liver histological features in most obese patients.

Key words: Non alcoholic fatty liver disease; Bariatric surgery; Obesity; Non alcoholic steatohepatitis

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INTRODUCTION

The term non alcoholic fatty liver disease (NAFLD) includes a spectrum of fatty liver diseases ranging from simple steatosis to steatohepatitis [non alcoholic steatohepatitis (NASH)] and cirrhosis[1]. The more progressive forms of NAFLD have been related to metabolic syndrome and obesity[2]. The epidemic of obesity has increased the prevalence of NAFLD and it is already the most common liver disorder in developed countries. Morbid obese patients have a high proportion of NAFLD. Most patients undergoing bariatric surgery have varying degrees of steatosis: as many as 36% have NASH and up to 4% have unsuspected cirrhosis. Only a small percentage of patients undergoing bariatric surgery have normal hepatic histology[3].

The optimal treatment of NASH has yet to be elucidated. In general, efforts have been developed to correct or improve insulin resistance and, in obese patients, weight loss has been prescribed. The effects of weight loss on NAFLD lesions have been studied and the reported effects of this therapy have been variable. Diet induced weight loss improved steatosis but did not always demonstrate an effect on histological parameters since in most studies repeated liver biopsy was not performed[3-8].

Also the effect of bariatric surgery could be confounding. Initially, it was described that rapid weight loss can exacerbate steatohepatitis in morbidly obese patients, especially after bariatric surgery[9]. This effect was more marked when malabsorptive procedures, like jejunoileal bypass or biliopancreatic diversion, were used. More recently, restrictive procedures, such as laparoscopic adjustable gastric banding, have demonstrated significant improvement in histopathological scoring[10-12], but in some studies the improvement in steatosis was accompanied by a progression of lobular inflammation[13].

We have less information about the effects on patients when mixed procedures were used[14]. The most frequently performed is Roux-en-Y gastric bypass, largely restrictive and mildly malabsorptive. In this procedure, the restriction is induced by a small neogastric pouch and a tight stoma and malabsorption by Roux-en-Y configuration of the small intestine[15]. The purpose of this study was to determine whether significant weight loss achieved through a standard mixed procedure - Roux-en-Y gastric bypass[16,17] - of bariatric surgery resulted in improvements in liver histopathology.

MATERIALS AND METHODS

Since May 2004, a prospective protocol has been followed for patients with morbid obesity that had a Roux-en-Y gastric bypass. Up to September 2005, twenty-six obese patients with a body mass index of more than 40 kg/m^2 who had significant medical, physical or psychosocial disabilities were considered for entry into the study. All patients underwent extensive preoperative assessment that included alcohol consumption, anthropometric measurements and laboratory tests. Laboratory tests included liver function tests, lipid profile, fasting plasma glucose, fasting insulin and hepatitis B and C serological analysis. Diagnosis of type 2 diabetes was based on the American Diabetes Association criteria[18]. Insulin sensitivity was estimated using the homeostatic model assessment method (HOMA)[19]. A diagnosis of metabolic syndrome was based on Adult Treatment Panel Ⅲ criteria[20]. At the time of the second biopsy, the clinical assessment and anthropometric and biochemical measures were repeated. Percentage of excess weight loss was calculated by dividing the weight change between paired biopsies by the excess weight before surgery, multiplied by 100.

Any patient with a history of alcoholism, consuming more than 200 g of alcohol per week, with evidence of hepatitis B or C or with a history of another specific liver disease, was included in the study.

An open Roux-en-Y gastric bypass with a modified Fobi-Capella technique (ring 7 cm; alimentary limb 225 cm; biliopancreatic limb 60 cm) was performed in all patients.

An index biopsy was taken at the time of surgery with a Hepafix needle. In all patients, a follow up biopsy was obtained as a percutaneous biopsy using a Hepafix needle. All biopsies were at least 2 cm in length and contained at least eight portal tracts. Informed written consent was also obtained from all patients at the time of the index biopsy as part of an approved prospective study of bariatric surgery. Informed written consent also was obtained from all subjects before the second biopsy and the study was conducted according to the ethical guidelines of the Helsinki Declaration.

All liver biopsy specimens were stained with hematoxylin eosin, picrosiriums for fibrosis and periodic acid Schiff (PAS) with diastase to help clarify the degree of inflammation.

Table 1 Criteria used for histological scoring[21]

Steatosis:
0 : None
1: up to 33%
2: 33%-66%
3: > 66%
Hepatocyte ballooning
0: None
1: Occasional, Zone 3
2: "Obvious" Zone 3
3: Marked, predominantly Zone 3
Mallory bodies
0: No Mallory bodies
1: Fewer than two in 10 to 20 × fields
2: More than two in 10 to 20 × fields
Glycogenated nuclei
0: Absent
1: Occasional
2: Several
Lobular inflammation (inflammatory foci per 20 × with a 20 × ocular)
0: None
1: 1 to 2/20
2: Up to 4/20
3: More than 4/20
Portal inflammation
0: None
1: Mild
2: Moderate
3: Severe
Fibrosis score:
Stage 0: No fibrosis
Stage 1: Zone 3 perisinusoidal/pericellular fibrosis; focally or extensively present
Stage 2: Zone 3 perisinusoidal/pericellular fibrosis with focal or extensive periportal fibrosis
Stage 3: Zone 3 perisinusoidal/pericellular fibrosis and portal fibrosis with a focal or extensive bridging fibrosis
Stage 4: Cirrhosis

A single hepatopathologist (HA), blinded to the patient, clinical and laboratory data and to whether the biopsy was the pre or post operative biopsy, examined all tissue sections at the same time and assessed liver histology using a systemic approach of necroinflammatory grading and fibrosis staging as described by Brunt *et al*[21] and modified by Kleine *et al*[22]. Individual histological features were observed and scored separately (Table 1): Steatosis: 0: None; 1: Up to 33%; 2: 33%-66%; 3: > 66%. Hepatocyte ballooning: 0: None; 1: Occasional, Zone 3; 2: "Obvious" Zone 3; 3: Marked, predominantly Zone 3. Mallory bodies: 0: No Mallory bodies; 1: Fewer than two in 10 to 20 × fields; 2: More than two in 10 to 20 × fields. Glycogenated nuclei: 0: Absent; 1: Occasional; 2: Several. Lobular inflammation (inflammatory foci per 20 × with a 20 × ocular): 0: None; 1: 1 to 2/20; 2: Up to 4/20; 3: More than 4/20. Portal inflammation: 0: None; 1: Mild; 2: Moderate; 3: Severe. Fibrosis score: Stage 0: No fibrosis; Stage 1: Zone 3 perisinusoidal/pericellular fibrosis; focally or extensively present; Stage 2: Zone 3 perisinusoidal/pericellular fibrosis with focal or extensive periportal fibrosis; Stage 3: Zone 3 perisinusoidal/pericellular fibrosis and portal fibrosis with a focal or extensive bridging fibrosis; Stage 4: Cirrhosis.

Finally, all were graded and staged for NASH according to the system proposed at the American Association for the Study of Liver Diseases single topic conference in September 2002[23].

Also, for each patient, the following variables were assessed at baseline and at the moment of second liver biopsy: age, waist circumference, weight, percentage excess weight loss, body mass index (BMI), alanine aminotransferase (ALT) level, aspartate aminotransferase (AST) level, gamma-glutamyl transferase level, bilirubin level, serum triglyceride level, cholesterolemia, serum high density lipoprotein cholesterol, serum low density lipoprotein cholesterol, blood glucose level, fasting insulin level, HOMA, steatosis amount, portal and lobular inflammation, fibrosis score and grade of NASH. The relationship between persistence of liver fibrosis after surgery (F > 1) and various risk factors was studied using a univariate analysis. In the univariate analysis, 2 groups were compared according to the presence or absence of significant fibrosis (F > 1) in the liver biopsy.

Statistical analysis of clinical and laboratory data was assessed using a paired samples *t* test. For histological comparisons pre-surgery and post-surgery, paired *t* tests were confirmed with Wilcoxon signed rank tests.

RESULTS

There were 26 patients (7 male and 19 female) with paired biopsies. Patient characteristics are shown in Table 2. There were no patients with cirrhosis and no complications from the gastric bypass or deaths during this study. The second biopsy was obtained 16.3 ± 3 mo (range: 12-22 mo) after bariatric surgery and no complications (bleeding, biloma, *etc*.) were observed during this postoperative percutaneous liver biopsy.

The percentage of excess weight loss was 72.1% ± 6.6% and the average rate of weight loss was 0.69 ± 0.22 kg/wk. Other clinical demographic and weight loss data are shown in Table 1. Weight loss was accompanied by significant favorable changes in anthropometric measures, significant decreases in blood pressure and major improvements in biochemical markers of metabolic syndrome, plasma glucose, insulin levels, insulin sensitivity and cholesterol levels (Table 1). Fifteen of the 26 patients (57.7%) fulfilled criteria for metabolic syndrome and only 2 (7.7%) fulfilled these criteria at the follow-up. Preoperatively, 7 (27%) patients had abnormal alanine aminotransferase or aspartate aminotransferase (> 0.58 μkat/L); postoperatively only 3 had abnormal alanine aminotransferase or an aspartate aminotransferse levels (> 0.58 μkat/L). There were no significant differences in aminotransferase levels with weight loss.

Histopathological results

Significant histopathological improvement was seen in steatosis ($P < 0.001$), ballooning degeneration ($P < 0.001$),

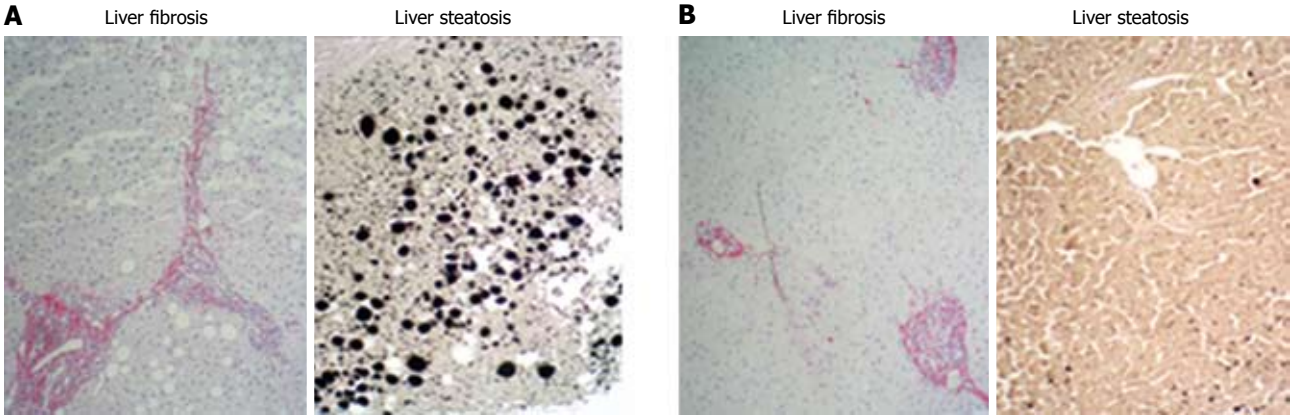

Figure 1 Marked improvement of liver fibrosis (left panel; picrosirius stain, ×10) and liver steatosis (right panel; osmium stain ×20) before (A) and after (B) bariatric surgery.

Table 2 Changes in anthropometric measurements and biochemistry between the patients' first and second liver biopsy

	Pre bariatric surgery	Post bariatric surgery	*P* value
Weight (kg)	130.8 ± 20.1	82.3 ± 13.7	< 0.001
BMI (kg/m^2)	49.3 ± 4.8	30.9 ± 4.3	< 0.001
% excess weight loss		72.1 ± 6.6	
Waist (cm)	137.1 ± 12.6	97.3 ± 11.0	< 0.001
Diabetic (%)	12 (46.1)	6 (23)	0.14
Hypertensive (%)	17 (65.4)	7 (26.9)	0.012
Metabolic syndrome	15 (57.7)	2 (7.7)	< 0.001
Cholesterol (mmol/L)	5.44 ± 1.00	4.29 ± 0.85	< 0.001
HDL-C (mmol/L)	1.24 ± 0.21	1.40 ± 0.19	0.005
LDL-C (mmol/L)	3.47 ± 0.76	2.46 ± 0.75	< 0.001
Triglycerides (mmol/L)	1.60 ± 0.63	0.93 ± 0.29	< 0.001
AST (μkat/L)	0.35 ± 0.09	0.36 ± 0.17	0.862
ALT (μkat/L)	0.49 ± 0.20	0.37 ± 0.31	0.143
GGT (U/L)	40.2 ± 17.4	19.2 ± 12.8	< 0.001
Fasting glucose (mmol/L)	6.46 ± 2.3	4.96 ± 0.55	0.001
Insulin (pmol/L)	235.2 ± 7.2	56.1 ± 7.2	0.006
Insulin resistance (HOMA)	9.99 ± 13.3	1.8 ± 1.4	0.006

BMI: Body mass index; HDL-C: High density lipoprotein cholesterol; LDL-C: Low density lipoprotein cholesterol; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; GGT: Gamma-glutamyl transferase; HOMA: Homeostatic model assessment method.

Table 3 Histological scores for the 26-paired biopsies

	Scores					*P* value
Feature	0	1	2	3	4	
Steatosis						< 0.001
Pre	0	13	8	5	-	
Post	24	2	0	0	-	
Ballooning degeneration						< 0.001
Pre	10	8	7	1	-	
Post	25	1	0	0	-	
Mallory bodies						0.005
Pre	18	8	0			
Post	26	0	0			
Glycogen nuclei						0.001
Pre	8	7	11			
Post	13	8	5			
Lobular inflammation						< 0.001
Pre	1	23	2	0		
Post	15	11	0	0		
Portal inflammation						0.05
Pre	1	23	2	0		
Post	7	19	0	0		
Fibrosis						0.001
Pre	1	17	6	2	0	
Post	9	12	4	1	0	

Mallory bodies ($P = 0.005$), glycogen nuclei ($P = 0.001$), lobular inflammation ($P < 0.001$), portal inflammation ($P = 0.005$) and fibrosis ($P < 0.001$) (Figure 1 and Table 3).

We classified subjects as having NASH if their biopsy scored at least 1 for both grade and stage. There were 25 patients (96%) with NASH in their index biopsy. By contrast, only 4 (15.3%) of the follow up biopsies demonstrated NASH ($P < 0.001$). Table 4 shows scoring for the grade and stage of NASH in liver biopsies performed at surgery and during follow up. None of the second biopsies revealed progression of grade or stage of liver disease.

The changes in steatosis are perhaps one of the more significant features in this analysis. Steatosis score improved overall by two or more grades in 12 patients and by one grade in 14 patients. Although portal inflammation improved significantly, it disappeared in only seven patients; after surgery, some degree of portal inflammation persisted in 19 patients.

Although the change in fibrosis was significant, it was not constant. Fibrosis score improved overall by two stages in 1 patient and by one stage in 10 patients. In 15 patients, fibrosis remained stable and we did not observe any patient with worsening of liver fibrosis (Figure 2). Eight patients had significant fibrosis (F > 1) before surgery. At the second liver biopsy, five patients still had significant fibrosis (Figure 3). In univariate analysis, patients with significant liver fibrosis after surgery had a significantly higher steatosis score, higher fibrosis score and lower AST level at the time of surgery than patients without (Table 5). After bariatric surgery, one of two patients with metabolic syndrome and none of the 6 patients with diabetes had a fibrosis score greater than 1 in the liver biopsy. There was no difference in the interval between

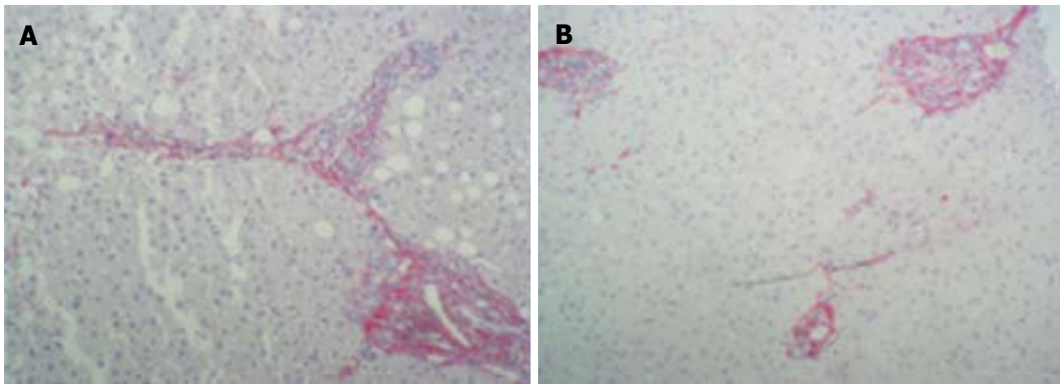

Figure 2 Persistence of liver fibrosis after bariatric surgery (picrosirius stain, ×10). A: At surgery; B: Post-surgery.

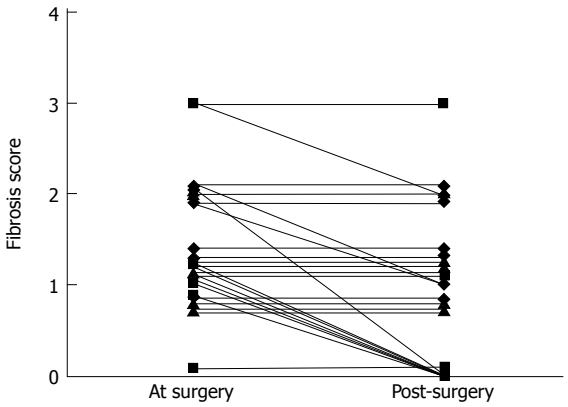

Figure 3 Evolution of fibrosis score in the liver biopsies of 26 patients with bariatric surgery.

surgery and biopsy in patients with or without significant fibrosis [432 ± 133 d *vs* 530 ± 122 d respectively, *P* = not significant (NS)]. Equally, there was no difference in the average rate of weight loss in patients with or without significant fibrosis (0.81 ± 0.26 kg/wk *vs* 0.66 ± 0.21 kg/wk respectively, *P* = NS).

DISCUSSION

In this study, we have demonstrated that, in patients with morbid obesity, weight loss induced by a mixed bariatric surgery (combination of malabsorptive and restrictive, Roux-en-Y gastric bypass with a modified Fobi-Capella technique) dramatically improved nonalcoholic fatty liver disease lesions observed in these patients.

There are various types of bariatric procedures. Initially, the most commonly used were primarily malabsorptive. Using these techniques, most of the obesity-related liver abnormalities did not improve, or even worsened. Jejunoileal bypass performed in the 1950-1970s was abandoned due to various complications, including significant hepatic lesions and even cirrhosis[24-26]. Numerous mechanisms, such as malabsorption of essential micronutrients, bacterial overgrowth and rapid weight loss with resulting massive influx of free fatty acids, have been implicated in the pathogenesis of NAFLD associated with jejunoileal bypass[27]. For example, Requarth *et al*[28] reported the long term morbidity after jejunoileal bypass in 453 patients and 24 of these developed acute liver failure (7%) and the 15 years probability of established cirrhosis was 8.1%.

More recently, restrictive or mixed techniques of bariatric surgery have been used. Lately, most of the published studies have focused on the effect of restrictive techniques on NAFLD. Thus, Dixon *et al*[10] used a laparoscopic adjustable banding technique that achieved a weight loss of around 50% and observed important improvements in steatosis, necroinflammatory changes and fibrosis.

The bariatric surgery technique used by us is mixed, restrictive and mildly malabsorptive. The techniques which, like ours, use a gastric bypass, produce a greater weight loss than gastric banding and also are longer lasting[29,30]. It would be interesting to know if these different types of surgical approaches are accompanied by similar effects on the treatment of liver lesions seen in obesity.

Using a bariatric surgery technique that combines restriction with mild malabsorption, we obtained a significant weight loss in patients. In addition, we have not only achieved a drastic remission of steatosis, but also improvements in lobular inflammatory activity and fibrosis. In our study, the percentage of excess weight loss (72%) was clearly superior to that observed in the Dixon study (52%), previously cited[10]. Although other studies have been done with techniques other than merely restrictive, good results have also been found in improvement of NAFLD[12,31].

It has been suggested that weight loss induced by malabsorptive procedures could disguise NASH improvements after bariatric surgery because, in some patients, impairment in liver fibrosis could be observed. This effect was observed in the study performed by Kral *et al*[32] that used biliopancreatic diversion. They found a constant improvement in metabolic syndrome but the effect on liver fibrosis varied. In their study, there was a frank improvement of fibrosis in patients with pre surgery advanced fibrosis (grades 1-2) and, in contrast, "*de novo*" fibrosis appeared in patients that did not have presurgery fibrosis. We have not observed this effect and the small number of samples in which we observed persistence of fibrosis was in those patients with a presurgery fibrosis greater than stage 1. The difference in the results can be

Table 4 Scoring for the grade and stage of non alcoholic steatohepatitis *n* (%)

	Scores				*P* value
	0	1	2	3	
Grade					
At surgery	1 (3.8)	12 (46.1)	11 (42.3)	2 (7.7)	0.001
Follow up biopsy	22 (84.6)	4 (15.4)	0 (0)	0 (0)	
Stage					
At surgery	1 (3.8)	17 (65.4)	6 (23.1)	2 (7.7)	0.001
Follow up biopsy	9 (34.6)	12 (46.1)	4 (15.4)	1 (3.8)	

Table 5 Factors associated with significant fibrosis (F > 1) after bariatric surgery

Variable	F 0-1 post surgery (*n* = 21)	F > 1 post surgery (*n* = 5)	*P* value
Mean steatosis score at surgery	1.5 ± 0.7	2.6 ± 0.5	0.002
Mean fibrosis score at surgery	1.1 ± 0.4	2.4 ± 0.5	< 0.001
Mean serum AST level at surgery (IU ± SD)	22.8 ± 11.6	11.6 ± 4.0	0.034

AST: Aspartate aminotransferase.

explained because Kral *et al*[32] found alcohol ingestion as a predictive factor of increasing fibrosis. Our series did not include alcoholic patients and this argues in favor of the fact that the increasing fibrosis seen in the Kral study was not related to the type of intervention, but to alcoholic ingestion post surgery.

In our study we saw a global improvement in portal fibrosis. Nevertheless, there were patients whose fibrosis did not improve; in 15 patients it remained stable. Barker *et al*[14] performed liver biopsy at surgery and postoperatively in 19 patients, mostly women without alcohol ingestion with a bariatric surgery technique similar to that used in our study, and found a frank improvement in fibrosis lesions, except in 4 patients whose lesions remained stable. Probably this slightly greater improvement in fibrosis can be explained by a longer interval between surgery and performance of the second biopsy. It was 21 mo in Barker's study and 16 mo in our work.

Similarly to Barker *et al*[14] and other studies about liver improvement of NASH with treatment[10,33], we also evidenced a persistence of portal inflammation. In most of our patients it was mild at surgery and significantly improved when it was analyzed globally; but in 19 patients some degree of portal inflammation persisted. This phenomenon is frequent and it has been suggested that these portal changes do not have a direct relationship with metabolic syndrome or insulin resistance[10,33].

When the factors that could predict persistence of significant liver fibrosis were analyzed, biochemical and clinical factors at the time of biopsies had low statistical power. The most important predictive factors were histological. Liver fibrosis and steatosis score at surgery were statistically associated with the persistence of significant fibrosis in liver biopsy post surgery. It has been demonstrated that steatosis at surgery and insulin resistance influence persistence of steatosis after bariatric surgery[34], but the influence of steatosis on the persistence of fibrosis has not been clearly demonstrated. In our work, we found that, not only liver fibrosis at surgery, but also steatosis score has an influence on the persistence of fibrosis. It is known that a fatty liver is more vulnerable to factors that lead to fibrosis[35] and in the presence of chronic liver diseases, steatosis may exacerbate liver injury[36]. The persistence of liver fibrosis in our patients mediated by various factors may be helped by the previous existence of a severe degree of steatosis.

Finally, patients with a persistence of significant fibrosis had a lower level of aminotransferases than those patients whose fibrosis was maintained or improved. This finding probably has low relevance, because in both groups aminotransferase levels were within normal range and it is also known that ALT values progressively decrease after BMI > 30 kg/m^2; therefore, it frequently happens that patients with morbid obesity have normal values of aminotransferase[27].

In conclusion, we have demonstrated that bariatric surgery, using a restrictive and mildly malabsorptive procedure, has a strong effect on improvement of liver abnormalities associated with non alcoholic fatty liver disease in the morbidly obese, although any significant changes were observed in aminotransferase enzymes.

COMMENTS

Background

Non alcoholic steatohepatitis (NASH) is one of the most common liver disease in patients with morbid obesity, and is associated with metabolic syndrome. The effects of the most current treatments for NASH (diet induced weight loss, bariatric surgery, *etc.*) are confounding. Therefore, it will be of interest to know the effects of Roux-en-Y gastric bypass on NASH.

Research frontiers

NASH is a very frequent disease affecting morbidly obese but it does not have an specific treatment to improve it, so the hotspot for NASH is to find a treatment for it.

Innovations and breakthroughs

The finding provides further evidence on the conclusions that Roux-en-Y gastric bypass associated weight loss enhances the resolution of metabolic syndrome and improves liver histological features in morbidly obese patients.

Applications

This paper, together with other related publications, can be collectively instructive to bariatric surgeons and nutritionists in their practice in treat morbidly obese patients.

Peer review

This paper shows interesting results even though the sample size is small. Perhaps the more interesting finding to highlight is the improvement in the fibrosis score after surgery. And it is a very interested study that discusses the benefits of surgically induced effects on obese patients with NAFLD.

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BRIEF ARTICLE

Efficacy of 3 years of adefovir monotherapy in chronic hepatitis B patients with lamivudine resistance

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Abstract

AIM: To study the effect of rescue monotherapy with adefovir (ADV) in patients with chronic hepatitis B (CHB) who developed drug resistance to lamivudine (LAM).

METHODS: A total of 76 treated CHB patients with resistance to LAM were enrolled in the present study. The patients' baseline characteristics, such as age, gender, blood tests and hepatitis B virus (HBV) DNA were collected; therapy duration and the response of each patient were also recorded. ADV monotherapy was set as the observation group A. Twenty-four patients with LAM resistance, who were set as group B, accepted combined therapy with LAM + ADV. Patients were followed up at 0, 12, 24, 52, 104 and 156 wk. Hepatitis B surface antigen status, hepatitis B e antigen (HBeAg)/anti-HBe status, HBV DNA level and biochemical indexes were monitored. Sequencer of HBV polymerase gene was performed on the ABI 3730 automated sequencer. If no desired effects had been achieved during the course of treatment, patients' choices were also taken into account. The control group was tested at the same time.

RESULTS: In the two groups, 27 cases developed viral breakthrough after LAM treatment response. The remaining 49 cases underwent biochemical rebound accompanied by rtM204I/V or rtL180M mutation. In group A, 52 cases finished 156 wk of ADV monotherapy; of whom, 36 cases were HBeAg positive and 16 HBeAg negative. In patients whose baseline HBV DNAs were 10^3-10^5 copies/mL, 88.8% of patients' HBV DNAs were lower than the lower test limit (10^3 copies/mL) after 12 to 156 wk of ADV treatment. In patients whose baseline HBV DNAs were $\geqslant 10^6$ copies/mL, 41.1%-47.0% of patients' HBV DNAs were lower than the lower test limit after the same course of ADV therapy (χ^2 were 4.35-5.4, 41.1%-47.0% *vs* 88.8% group 10^3-10^5 copies/mL, $P < 0.01$). In group A, seroconversion of HBeAg developed in 8 of 36 cases (22.2%). In group B, 24 cases finished 156 wk of LAM + ADV; of whom, 17 cases were HBeAg positive and 7 HBeAg negative. In patients whose baseline HBV DNAs were 10^3-10^5 copies /mL, 81.8% of patients' HBV DNAs were lower than the lower test limit (10^3 copies/mL) after 12 to 156 wk of treatment. In the patients whose baseline HBV DNAs were $\geqslant 10^6$ copies/mL, 46.1%-53.8% of patients' HBV DNAs were lower than the lower test limit after the same course of LAM + ADV therapy (χ^2 were 4.1-5.0, 46.1%-53.8% *vs* 81.8% group 10^3-10^5 copies/mL, $P < 0.05$-0.01). In group B, 4 of 17 cases (23.5%) developed seroconversion of HBeAg. Treatment outcomes in groups A and B were comparable.

CONCLUSION: In both group A and B, the ratios of virological response have similar efficacy in patients with lower baseline HBV DNAs.

Key words: Adefovir; Lamivudine; Drug resistance; Chronic hepatitis B; Antiviral therapy; Monotherapy

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INTRODUCTION

The main treatment for chronic hepatitis B (CHB) is antiviral therapy. Nucleoside analogues are one of the major choices. However, drug resistance hinders nucleoside analogues from a wide application, especially in cases of long-term oral administration. When multiple drugs are used, the occurrence of single or multiple drug resistance is an issue that cannot be neglected. Considering the need for nucleoside administration in the long run, an appropriate strategy is necessary so that multiple drug resistance can be avoided.

Lamivudine (LAM) resistance is fairly common in clinical practice. The present study was performed in order to evaluate the efficacy of single rescue therapy with adefovir (ADV). We hope to determine (1) the feasibility of single agent salvage therapy with ADV in LAM resistant cases; and (2) whether a predicted efficacy can be derived from the baseline hepatitis B virus (HBV) DNA load.

MATERIALS AND METHODS

Patients

During the period between April 2006 and March 2007, there were 85 cases of patients who failed the initial LAM treatment in our hospital. Among those cases, 27 cases developed viral breakthrough after LAM treatment response. Viral breakthrough was defined as serum HBV DNAs rebound greater than 10^2 copies/mL. The remaining 49 cases underwent biochemical rebound accompanied by rtM204I/V or rtL184M mutation. In group A, 52 cases were employed and oral ADV in 10 mg/d was given for 8 wk before the cessation of LAM. In group B (24 cases), ADV of 10 mg/d and LAM of 100 mg/d were administered. Diamine glycyrrhizin or silymarin was used for patients with elevated alanine aminotransferase (ALT). Patients were excluded if they had the following: interferon administration in the last 6 mo; combined infection of HCV or HDV; autoimmune hepatitis; elevated confirmed liver cancer or suspected liver cancer; decompensated liver disease; and a history of alcohol abuse, pregnancy or breast-feeding. The diagnosis was made according to the guidelines[1]. There was no history of immunomodulatory agent administration in the last 6 mo. All patients gave informed consent before treatment. Finally, 52 patients were enrolled in ADV single drug and 24 patients were enrolled ADV plus LAM in this prospective research. In patients with HBeAg-positive CHB, combined response was defined as ALT levels returning to normal, accompanied by undetectable HBV DNA and HBeAg seroconversion; partial response was defined as ALT levels returning to normal, HBV DNA < 10^3 copies/mL with no seroconversion.

Table 1 Baseline demographic and virological data of the study population

Factor	A group *n* (range)	B group *n* (range)
Sex (M:F)	38:14	18:6
Age (yr)	27.6 (19-60)	25.7 (18-59)
ALT (U/L)	165 (44-1000)	230 (110-640)
HbeAg positive/HBeAg negative	36:16	17:7
HBV DNA (log copies/mL)	7.35 (3.00-9.0)	7.5 (3.0-9.0)
LAM-induced mutation of HBV DNA and rebound	40:12	20:4

Continuous variables were expressed as median (range). M: Male; F: Female; ALT: Alanine aminotransferase; HBV: Hepatitis B virus; HbeAg: Hepatitis B e antigen; LAM: Lamivudine.

Monitoring of patients

Patients were followed up at 12, 24, 52, 104 and 156 wk. Hepatitis B surface antigen status, hepatitis B e antigen (HBeAg)/anti-HBe status, HBV DNA level and biochemical indexes were monitored. If no desired effects had been achieved during the course of treatment, patients' choices were also taken into account. Group B was tested at the same time.

Sequencing of HBV polymerase gene and assay of HBV DNA

Sera from patients on presentation were taken for the following tests: (1) sequencing of HBV polymerase gene, which was performed on the ABI 3730 automated sequencer; and (2) assay of HBV DNAs, which was determined by quantitative fluorescence polymerase chain reaction (PCR) on the ABI 7000 (Applied Biosystems) with a lower limit of detection of 1000 copies/mL. HBV DNAs levels lower than the detection limit were regarded as negative for statistical calculations.

RESULTS

Demographics

A total of 76 CHB patients were enrolled. The baseline demographics, liver function tests, liver biochemistry, HBV Gene sequencing determination data and HBV DNAs levels are listed in Table 1.

Baseline characteristics and treatment outcomes

At 12, 24, 52, 104 and 156 wk, there was no statistical difference in the number of patients who achieved virological response (VR, defined as HBV DNA < lower

Table 2 Control of the therapeutic effect in the two groups

Group	Baseline (copies/mL)	*n*	12 wk		24 wk		52 wk		104 wk		156 wk	
			VR[1]	BR[2]	VR[1]	BR[2]	VR[1]	BR[2]	VR[1]	BR[2]	VR[1]	BR[2]
A	10^3-10^5	18	16 (88.8)[1]	16 (88.8)	16 (88.8)[1]	16 (88.8)	16 (88.8)[3]	16 (88.8)	16 (88.8)[1]	16 (88.8)	16 (88.8)[1]	16 (88.8)
A	$\geqslant 10^6$	34	14 (41.1)	24 (70.5)	15 (44.1)	30 (88.2)	16 (47.0)	22 (64.7)	16 (47.0)	26 (76.4)	16 (47.0)	18 (52.9)
B	10^3-10^5	11	9 (81.8)[4]	9 (81.8)	9 (81.8)[4]	10 (90.9)	9 (81.8)[4]	10 (90.9)	8 (72.7)	9 (81.8)	9 (81.8)[4]	9 (81.8)
B	$\geqslant 10^6$	13	6 (46.1)	10 (76.9)	6 (46.1)	10 (76.9)	6 (46.1)	9 (69.2)	7 (53.8)	9 (69.2)	6 (46.1)	9 (69.2)
A	HBeAg positive	36	18 (50.0)	26 (72.2)	20 (55.5)	30 (83.3)	20 (55.5)	26 (72.2)	22 (61.1)	26 (72.2)	20 (55.5)	22 (61.1)
A	HBeAg negative	16	12 (75.0)	14 (87.5)	14 (87.5)	16 (100.0)	12 (75.0)	12 (75.0)	14 (87.5)	16 (100.0)	12 (75.0)	6 (75.0)
B	HBeAg positive	17	8 (47.8)	12 (70.5)	9 (52.9)	15 (88.2)	9 (52.9)	15 (88.2)	9 (52.9)	13 (76.4)	10 (58.8)	11 (64.7)
B	HBeAg negative	7	5 (71.4)	6 (85.7)	5 (71.4)	6 (85.7)	6 (85.7)	6 (85.7)	5 (71.4)	6 (85.7)	5 (71.4)	6 (85.7)
A	ALT < 2 × ULN	20	17 (85.0)	18 (90.0)	16 (80.0)	18 (90.0)	14 (70.0)	14 (70.0)	14 (70.0)	18 (90.0)	14 (70.0)	14 (70.0)
A	ALT > 2 × ULN	32	18 (56.2)	22 (68.7)	18 (56.2)	28 (87.5)	18 (56.2)	26 (81.2)	22 (68.7)	24 (75.0)	18 (56.25)	20 (62.5)
B	ALT < 2 × ULN	10	8 (80.00)	9 (90.0)	8 (80.0)	10 (100.0)	8 (80.0)	10 (100.0)	8 (80.0)	10 (100.0)	8 (80.0)	9 (90.0)
B	ALT > 2 × ULN	14	8 (57.1)	10 (71.4)	8 (57.1)	12 (85.7)	10 (71.4)	12 (85.7)	10 (71.4)	12 (85.7)	10 ((71.4)	12 (85.7)

Group A: Treated with adefovir; Group B: Treated with adefovir and lamivudine; [1]VR: Defined as HBV DNA < lower detection limit; [2]BR: Defined as ALT < lower detection limit; [3]$P < 0.01$ ($\chi^2 = 4.35$-5.4) *vs* group A with baseline HBV DNA $\geqslant 10^6$ copies/mL; [4]$P < 0.05$ ($\chi^2 = 4.1$-5.0) *vs* group B with baseline HBV DNA $\geqslant 10^6$ copies/mL. VR: Virological response; BR: Biochemical response; HBV: Hepatitis B virus; HBeAg: Hepatitis B e antigen; ALT: Alanine aminotransferase.

Table 3 Response to adefovir salvage therapy *n* (%)

Project		HBV DNA (copies/mL)			HBeAg positive		rtA181V/S variation
		10^3	10^3-10^6	$\geqslant 10^7$	HBeAg seroconversion	HBeAg elimination	Change drug
Baseline level	A	12 (23.0)	22 (42.43)	18 (34.6)	36		
	B	5 (20.8)	11 (45.8)	8 (33.3)	17		
12 wk	A	30 (57.7)[1]	18 (34.6)	4 (7.6)[2]	0 (0)	2 (5.5)	
	B	14 (58.3)[4]	8 (33.3)	2 (8.3)[5]	1 (5.8)	1 (5.8)	
24 wk	A	32 (61.5)[4]	20 (38.4)	0 (0)[3]	4 (11.1)	2 (5.5)	2 (5.5)
	B	15 (62.5)[4]	8 (33.3)	1 (4.1)[5]	2 (11.7)	2 (11.7)	1 (5.8)
52 wk	A	34 (65.4)[1]	18 (34.6)	0 (0)[3]	4 (11.1)	2 (5.5)	2 (5.5)
	B	16 (66.6)[4]	8 (33.3)	0 (0)[5]	4 (23.5)	2 (11.7)	1 (5.8)
104 wk	A	36 (69.2)[1]	16 (30.7)	0 (0)[3]	8 (22.2)	4 (11.1)	2 (5.5)
	B	16 (66.6)[4]	8 (33.3)	0 (0)[5]	4 (23.5)	2 (11.7)	1 (5.8)
156 wk	A	34 (65.4)[1]	16 (30.7)	2 (3.8)[3]	8 (22.2)	6 (16.6)	4 (11.1)
	B	15 (62.5)[4]	8 (33.3)	1 (4.1)[5]	4 (23.5)	2 (11.7)	2 (11.7)

Group A: Treated with adefovir; Group B: Treated with adefovir and lamivudine; [1]$P < 0.001$ ($\chi^2 = 6.4$-11.0) *vs* group A baseline HBV DNA 10^3 copies/mL; [2]$P < 0.05$ ($\chi^2 = 4.7$); [3]$P < 0.01$ ($\chi^2 = 7.9$-10.8) *vs* group A baseline HBV DNA 10^7 copies/mL; [4]$P < 0.001$ ($\chi^2 = 5.9$-11.7) *vs* group B baseline HBV DNA 10^3 copies/mL; [5]$P < 0.01$ ($\chi^2 = 7.7$-11.0) *vs* group B baseline HBV DNA 10^6 copies/mL. HBV: Hepatitis B virus; HBeAg: Hepatitis B e antigen.

detection limit) or biochemical response (defined as ALT < lower detection limit) between the two groups with a baseline of HBeAg positive and HBeAg-negative. More than that, there was also no statistical difference between the two groups with a baseline ALT < 2 × upper limit of normal (ULN) and ALT > 2 × ULN. However, patients with a lower baseline HBV DNA level tended to have a higher VR ratio. Baseline HBV DNAs, HBeAg status, ALT and their relationship to treatment outcomes of each group are shown in Table 2.

HBeAg seroconversion state in treatment course with ADV monotherapy

As shown in Table 3, after 12 wk of treatment, HBeAg seroconversion was 0/36 cases in group A. At 24 and 52 wk, it was 4/36 cases (11.1%). After 104 and 156 wk of treatment, the HBeAg seroconversion ratio rose in 8/36 cases (22.2%). After 12 to 52 wk of treatment, HBeAg turned negative in 2/36 cases (5.5%). After 156 wk of treatment, HBeAg turned negative in 6/36 cases (16.6%). The ratio of HBV DNAs lower than 10^3 copies/mL gradually increased with the treatment course when compared with the baseline (χ^2 were 6.4-11.0, 23.05% *vs* 65.4%, $P < 0.01$). After treatment, the ratio of HBV DNAs > 10^7 copies/mL decreased gradually with the treatment course (χ^2 were 4.07-10.8, 3.8% *vs* 34.6%, $P < 0.05$-0.01). No significant difference was observed in either group.

The handling of poor effect and viral rebound

During treatment, 10 cases underwent poor response or viral rebound. The rtA181V/I/S loci variation was detected in 4 cases (7.6%). Entecavir (ETV) was added at 52 and 108 wk. After one year, the 2nd HBV polymerase gene sequencing was performed and rtM204I variation

persisted in 2 cases (2/10). Although ALT turned to normal, HBV DNAs were higher than 10^3 copies /mL in the time course. Six patients shifted to ETV at 24 wk. At 104 wk, 10 patients' ALT turned normal with HBV DNAs < 10^3 copies/mL. However, after self-withdrawal at 4-12 wk, ALT became abnormal. In group B, 3 patients (12%) at 104 and 156 wk respectively were detected with rtA181V/I/S and rtM204I, rtL180M sites of variation.

DISCUSSION

In the treatment of chronic hepatitis B, ADV tends to induce mutations of the HBV genome that are different from that of LAM. Thus, ADV may serve as an alternative for the patients with LAM resistance. Due to the earlier entry of LAM into China's market, ADV used to act as a salvage therapy in patients who could not gain a satisfying response or have resistance to LAM. Several reports have indicated that combined use of ADV and LAM surpasses single salvage treatment with ADV[2-6]. However, the enrolled patients were limited. More than that, expenditure and multi-drug resistance are worthy of care[7,8]. Thereupon, we executed single salvage treatment of ADV in 2006 in patients who developed resistance to LAM. Similarly to the observation of Shin *et al*[9], Lee *et al*[10] and Kim *et al*[11] patients who have a lower viral load tended to have a higher response ratio to ADV monotherapy. We also observed that patients who have a higher viral load usually have a poor response, which agreed with Idilman *et al*[12], Chen *et al*[13] and Aizawa *et al*[14]. Unauthorized withdrawal of drugs would lead to the rebound of HBV DNA. In the cases where HBV DNAs cannot return to normal, the ratio of ALT normalization was also low. After 156 wk of treatment, the ratio of HBeAg seroconversion was 22.2% (8/36 cases), which was similar to both Aizawa *et al*[15], Ryu *et al*[16] and Heo *et al*[17] of single rescue therapy of ADV in patients who developed drug resistance to LAM and the ratio of HBeAg seroconversion in patients who accepted single ADV treatment for the first time. After ADV salvage therapy, the proportion of patients whose HBV DNAs were lower than 10^3 copies/mL increased gradually with time compared with the baseline. The number of patients whose HBV DNAs were higher than 10^7 copies/mL decreased gradually with time compared with the baseline (Table 2). The overall decline of HBV DNA load was similar to that in the initial treatment with ADV[18]. Variation of rtA181V/I/S loci was detected in 4 cases (7.6%) after 104 wk of treatment. Among them, 2 cases (3.8%) remained with rtM204I loci variation after 52 wk of ADV treatment. Meanwhile, the cases remained in a state of low HBV DNAs load and ALT were stable. Mutation rate was similar to that in the report of initial treatment of ADV. No multiple resistance sites were screened out. In group A, HBV DNA was slightly higher than group B. However, it was more difficult to deal with multiple points of resistance.

Our results showed that single salvage therapy of ADV has a certain effect on patients who developed resistance to LAM. We think it is suitable to initiate ADV salvage therapy in patients whose HBV DNA is in 10^3-10^5 copies/mL, although timely monitoring on HBV DNA load is needed. It is sagacious to adopt combination therapy if no satisfying effect is achieved. Chen *et al*[19] has also observed that no additional benefit may be gained in initiating combination therapy at the very beginning[20,21]. HBV DNA load at 24 wk or 12 mo may serve as a predictor of ADV resistance[22-25]. Single salvage therapy with ADV is conducive to reducing non-individualized combination therapy, the potential hazards of multiple drug resistance, the financial burden and mental stress of patients due to long-term additional medication and to enhance compliance. ADV rescue treatment cannot gain a satisfying effect in LAM resistant cases which may stem from the limitation of ADV in patients with high HBV DNA loads. No significant difference was observed in the two groups. Timely detection of HBV DNA load, virological breakthrough and genome variation are necessary for the achievement of a preferable response. In the patients who do not have desired outcome, combined therapy may be a suitable strategy.

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COMMENTS

Background

The greatest challenge in the treatment of hepatitis B with a nucleoside was drug resistance after long-term treatment. At the same time, with the multi-drug combination, resistance to the multi-drug is also a matter of time. Due to the earlier entry of resistance to lamivudine (LAM) into China's market, adefovir (ADV) is used as a salvage therapy in patients who cannot gain a satisfying response or have resistance to LAM. Several reports have indicated that the combined use of ADV and LAM surpasses single salvage treatment with ADV. However, hepatitis B virus (HBV) DNA may have a significant influence on the antiviral effect of ADV. The present research aims at evaluating the effect of ADV monotherapy on LAM resistant patients with diverse levels of HBV DNA.

Research frontiers

How to control and reduce the occurrence of nucleoside resistance is a current difficulty and hot issue. Particularly, it is great challenge when multiple drug and long term administration are needed. To date, there is no satisfying solution for this issue.

Innovations and breakthroughs

Although there is debate on whether ADV or ADV plus LAM should be used for the rescue treatment on LAM resistance, HBV DNA level may have a significant effect on the efficacy of salvage therapy. Sagacious and individualized choice should be made for patients with different HBV DNA levels.

Applications

Although multicenter and randomized observations are needed, the present research may shed light on the strategy of rescue therapy on LAM resistance.

Terminology

Salvage therapy: A therapeutic approach, involving chemotherapy, radiation therapy or surgery, after initial regimens have failed to lead to improvement in a patient's condition. Salvage therapy is most often used for neoplastic diseases; Drug resistance: Diminished or failed response of an organism, disease or tissue to the intended effectiveness of a chemical or drug.

Peer review

The authors' aim is important, although it is difficult to understand how the

authors selected observation and control groups among chronic hepatitis B patients. The authors prospectively evaluated the effectiveness of ADV *vs* ADV + LAM treatment in patients with lamivudine resistance. They concluded that combination therapy should be used in patients with high DNA levels. This conclusion has practical implications.

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CASE REPORT

Acute abdomen and ascites as presenting features of autosomal dominant polycystic kidney disease

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Abstract

We describe a patient with sudden onset of abdominal pain and ascites, leading to the diagnosis of autosomal dominant polycystic kidney disease (ADPKD). Her presentation was consistent with acute liver cyst rupture as the cause of her acute illness. A review of literature on polycystic liver disease in patients with ADPKD and current management strategies are presented. This case alerts physicians that ADPKD could occasionally present as an acute abdomen; cyst rupture related to ADPKD may be considered in the differential diagnoses of acute abdomen.

Key words: Autosomal dominant polycystic kidney disease; Acute abdominal pain; Ascites; Polycystic liver disease

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INTRODUCTION

Sudden onset of abdominal pain and ascites usually signify critical illness often requiring urgent intervention. We describe a patient with such presentation where investigations led to an unanticipated diagnosis of autosomal dominant polycystic kidney disease (ADPKD), a hereditary disease characterized by adult onset progressive development and enlargement of cysts in the kidneys and other organs including the liver. It has been known that clinical presenting features and manifestations at the early stages of ADPKD are mostly insidious[1,2]. Acute abdomen with ascites as a presenting feature of ADPKD has not been previously described.

CASE REPORT

A 45-year-old female presented to a local emergency department (ED) with complaints of acute onset upper abdominal pain and distension associated with nausea and vomiting.

Her medical history included well-controlled hypertension, hyperlipidemia and hypothyroidism for approximately two years. Her medications included daily enalapril 10 mg, rosuvastatin 10 mg, levothyroxine 50 mg and vitamin D supplement. She worked full-time as a physician and was mother to five children (ages 5 to 14 years) with the last two children being twins. Her pregnancies were uneventful except for the last pregnancy (with the twins) about six years ago; she had transient thrombocytopenia and mild liver enzyme elevation prior to

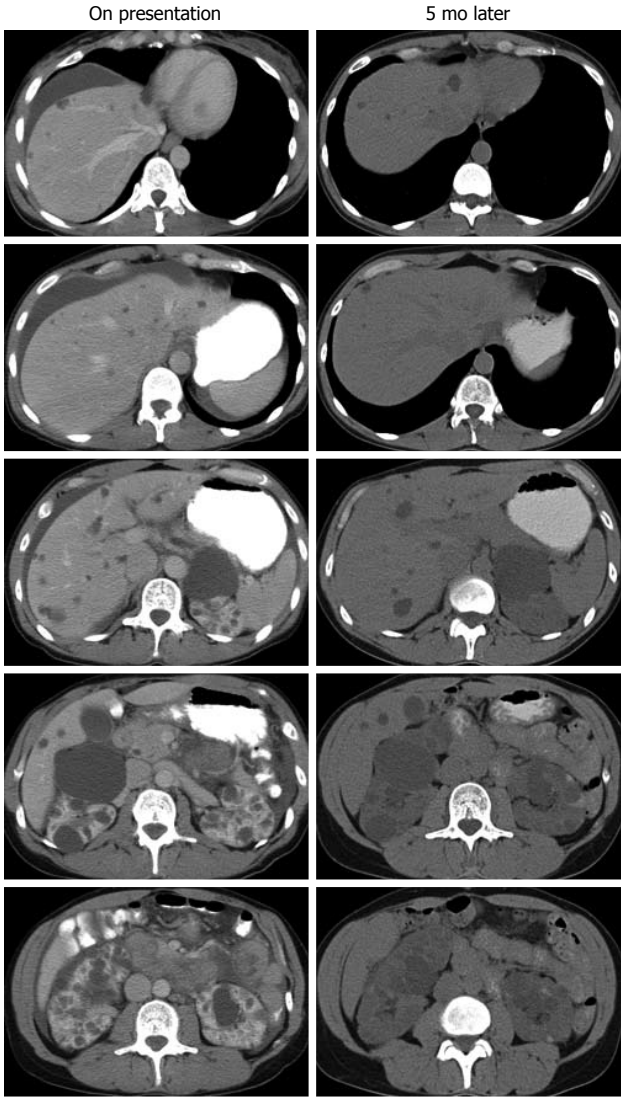

Figure 1 Contrast-enhanced abdominal computed tomography. Left panel: Serial images from an axial computed tomography (CT) scan at the time of the patient's presentation to the local emergency department, showing perihepatic ascites and multiple liver and kidney cysts; Right panel: Repeat abdominal CT scan five months following the acute presentation, showing corresponding images of multiple liver and kidney cysts without perihepatic ascites.

the delivery which resolved promptly after the delivery. Reports of ultrasound examinations during her pregnancies did not indicate presence of hepatic or renal cysts. She had been on hormonal contraceptives intermittently with a cumulative duration of about 20 years. She denied a known family history of ADPKD.

On the day of her presentation to the local ED, while at work, she developed a sudden-onset sharp upper abdominal pain, associated with shortness of breath and a feeling of abdominal fullness. She felt nauseated and vomited several times. She was rushed to the ED, where her vital signs were found to be normal. Her abdomen was mildly distended and tender on palpation in the upper quadrants with some guarding. Laboratory studies showed hemoglobin 12.9 mg/dL, leukocytes 7.3 × 10^9 cells/mL, albumin 4.2 mg/dL, aspartate aminotransferase 26 U/L, alanine aminotransferase 45 U/L, alkaline phosphatase 66 U/L, bilirubin 0.6 mg/dL, blood urea nitrogen 19 mg/dL, creatinine 1.1 mg/dL, and urinalysis was unremarkable. Her serum amylase and lipase were normal. Ultrasonography of the abdomen revealed perihepatic ascites, multiple cysts throughout the liver with the largest cyst being 1.7 cm in diameter, and numerous cysts in the kidneys. Contrast-enhanced abdominal computed tomography (CT) confirmed perihepatic ascites and the benign-appearing cysts in the liver and kidneys (Figure 1, left). She was hospitalized for monitoring and treated with intravenous fluids and antiemetics (ondansetron). The following day, an esophago-gastro-duodenoscopy with random biopsy was performed which was without abnormality. Her pain, nausea and vomiting subsided, and she was discharged. A week later, she underwent an elective laparoscopic cholecystectomy. Intraoperative inspection showed no abnormality except for the visible fluid-filled hepatic cysts. The pathology of the gallbladder was normal.

Five months later, she presented to our institution for a second opinion. She was asymptomatic and physical examination was unremarkable. Her medications were unchanged. An abdominal CT scan showed both liver cysts and bilateral kidney cysts (Figure 1, right), consistent with ADPKD. In retrospect, her acute abdomen and ascites were consistent with hepatic cyst rupture.

DISCUSSION

In this patient, the acute abdomen with ascites was the presenting feature of what turned out to be ADPKD. ADPKD in her was diagnosed based on the findings of numerous fluid-filled cysts in bilateral kidneys and liver, and the absence of features to suggest any alternative diagnosis. She did not have a positive family history which could be consistent with the general observation of de novo *PKD* gene mutations in a minority of ADPKD patients (5%-10%)[3]. Gene based diagnostic study is not required as the clinical presentation and radiographic findings are the gold standard for establishing a diagnosis[4].

Although all ADPKD patients develop kidney cysts, at the early stages of the disease (when the size of the affected organs are not significantly enlarged), the majority of the patients are asymptomatic or symptoms are so mild that often go unnoticed, such as reduction in urine concentration capacity. In a case series of 171 ADPKD patients, symptoms that led to investigation and ultimate diagnosis only accounted for 37.4% of the patients[5]. The most common symptoms were back pain (17.4%), gross hematuria (16.4%) and non-specific abdominal pain (16.4%). Although known to occur rarely in ADPKD patients with late stage cystic disease and kidney failure[6,7], liver cyst rupture leading to acute abdomen and ascites as initial symptoms of ADPKD has not been previously described.

Polycystic liver disease (PLD) is the most frequent extra-renal manifestation in ADPKD, yet, it is clinically

silent in majority of cases and only infrequently medical attention is needed. The following is an overview on its natural history, complication, pathogenesis, and treatment strategies.

Natural history and complications of PLD

Liver cysts usually start to appear after ADPKD patients reach puberty; by age 30 years, up to 94% of affected individuals have detectable PLD by imaging studies[8,9]. With age, almost all ADPKD patients have varying degrees of PLD. Significant variations can occur, even among affected individuals from the same family. However, for each patient, liver cysts grow steadily over time in both number and size. Although liver cysts may be innumerable, majority (approximately 80%) of the patients remain asymptomatic[10]. A minority of patients with a few large dominant cysts or with severe cystic liver enlargement develop symptoms, including pain from cyst growth, cyst hemorrhage, cyst infection and symptoms of compression to adjacent organs due to mass effects from cystic liver. It has been observed that ADPKD patients on dialysis or following transplantation are more likely to develop symptoms resulting from the mass effect or from cyst-related complications such as rupture, hemorrhage, or infection[8]. Spontaneous cyst rupture into the peritoneal cavity is extremely rare.

Pathogenesis of PLD

Although ADPKD gene mutations are well known to cause cystic liver phenotype, the precise pathogenesis for the development and enlargement of liver cysts has not been fully elucidated. Morphological studies of individual liver cysts reveal that cysts originate from biliary microhamartomas (also termed Von Meyenburg's complexes that arise from proliferation of biliary ductules)[11] and from peribiliary glands[12]. Liver cysts are lined with epithelium of biliary origin[13] and, with progressive growth, cysts become detached from their origins. It is believed that the liver cyst growth is attributable to concerted effects of proliferation in cyst-lining epithelia, solute and fluid secretion into the cysts, remodeling of cyst-surrounding matrix and neovascularization[14].

Estrogen has been shown to influence the development and progression of liver cysts[15]. Biliary epithelia (cholangiocytes) and cyst-lining cells in ADPKD, in contrast to normal liver parenchymal cells, express estrogen receptors aberrantly[16]. Estrogen is able to act directly through estrogen receptors and indirectly by potentiating the effects of growth factors to promote cholangiocyte proliferation and secretion[17]. Moreover, through potentiating the effects of vascular endothelial growth factor, estrogen promotes adaptive angiogenesis, vital for cyst growth[18]. Estrogen therefore affects multiple aspects in promotion of cyst growth. Consistent with these data, severe degree of cystic liver enlargement occurs mostly in female patients, especially in multiparous women and women on oral contraceptive or estrogen replacement therapy[15,19]. Our patient had multiple pregnancies and had also been on hormonal contraceptive for many years. It is tempting to speculate that her estrogen exposure over the years might have contributed to her cystic liver disease and her dramatic presentation.

Diagnosis of PLD

PLD is diagnosed by imaging studies, including ultrasound, CT, and magnetic resonance imaging (MRI). Serum biochemical profile is typically normal and synthetic functions of the liver are preserved in virtually all cases. The only laboratory abnormalities seen in severe PLD are mild elevations of γ-glutamyltransferase and alkaline phosphatase. Among imaging studies, ultrasound is preferred because of its low cost and lack of radiation. However, CT and MRI are more sensitive and accurate in detecting the number and size of liver cysts.

PLD should be differentiated from simple liver cysts, which often occur in normal individuals with age (up to four cysts at age 60 years)[15]. PLD should also be differentiated from occasional cysts associated with autosomal recessive polycystic kidney disease (ARPKD). ARPKD is a rare (1:20 000) disease with congenital hepatic fibrosis as its major hepatic manifestation. Occasionally, liver cysts in PLD may also be confused with cystadenomas[20], especially when cysts contain hemorrhagic fluids. In such cases, further investigation and close follow-up are necessary. In our patient, there were no clinical or imaging evidences of these conditions.

Treatment of PLD

Most cases of PLD require no treatment. Symptomatic PLD requires interventions to reduce cyst volume and liver size. To date, apart from avoidance of estrogen, no specific medical regimen has been established to halt the PLD development or to retard PLD progression. Invasive management strategies including percutaneous cyst aspiration with or without sclerosis, laparoscopic cyst fenestration, combined liver resection and cyst fenestration, and rarely, liver transplant have been the treatment modalities, aimed to palliate symptoms[21].

Percutaneous cyst aspiration and sclerosis under ultrasound or CT guidance is an effective modality to treat large dominant cysts that are not numerous. Cyst aspiration alone is often carried out diagnostically to determine whether there is a direct correlation between the cysts and the patient's symptoms. Without the sclerosing therapy, however, cysts often re-expand in weeks to months following the procedure. Sclerosing therapy reduces the possibility of cyst re-expansion. Sclerosing therapy constitutes injection of an appropriate volume (approximately 25% of the aspirated cyst fluid volume) of 95%-99% ethanol or acidic solutions of tetracycline or minocycline into the cyst following cyst fluid aspiration. The patient then assumes different physical positions to ensure a maximum contact between the sclerosing solution and cyst-lining epithelia. The sclerosing fluid is then aspirated. This method carries approximately 70%-90% success rate of cyst obliteration[22]. For cysts with a di-

ameter > 10 cm, repeat aspiration and sclerosis may be necessary for a sustained cyst obliteration[23].

More invasive surgical interventions are reserved for patients with severely symptomatic hepatomegaly due to PLD. Schnelldorfer *et al*[21] retrospectively studied 141 patients with PLD who underwent partial hepatic resection with remnant cyst aspiration, cyst fenestration alone, or orthotopic liver transplantation for symptoms or complications related to PLD at Mayo Clinic Rochester. Based on the experience, they propose to devise treatment plans for PLD patients on the basis of their clinical and radiographic features. They have classified PLD patients into four types, types A to D. Patients with no clinical symptoms or mild symptoms are classified as type A and no surgical treatment is indicated. Patients with moderate to severe symptoms with large-sized dominant cysts and preservation of at least two sectors of normal liver parenchyma are classified as type B and they should be considered for cyst fenestration. Those with severe symptoms associated with enlarged liver but having more than one sector of normal liver parenchyma are classified as type C for whom partial liver resection with remnant cyst fenestration may be considered. Patients with severely enlarged liver and with little normal liver parenchyma are classified as type D and the only treatment option for these patients is liver transplant. Although these operative treatments offer sustained improvement in performance and health status, they are technically demanding and perioperative complications are substantial. For instance, liver resection with remnant cyst fenestration carries overall morbidity of 60%-70%, including biliary leak and ascites. For liver transplant, survival rate seems lower than in non-PLD patients with liver transplant. Thus, for individual patients, treatment choices depend on local expertise, which is one of the most critical factors that predict success. Referral to a tertiary center with adequate expertise would more likely result in an optimal treatment outcome.

In summary, although typically asymptomatic, a subset of patients with PLD related to ADPKD may present with abdominal discomfort. The degree of symptoms depends on the extent and rapidity of liver cyst growth. As shown in this case, sudden rupture of hepatic cyst can occur and be an initial presenting feature of PLD. Though not previously described, such an occurrence is not entirely surprising, as rupture can conceivably occur in expanding superficial cysts. Thus, PLD and ADPKD should be considered as one of the differential diagnoses in patients with such a presentation. Conservative management, at least in this case, seemed to have sufficed. Whether such rupture would recur is uncertain and warrants ongoing follow up.

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CASE REPORT

Estrium Whey induced hepatitis in a patient with metastatic breast cancer: Case report

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Abstract

Estrium Whey is an alternative nutritional support therapy for women. It's enhanced with specific nutrients including phytoestrogens, folate, antioxidants and fiber to support healthy estrogen detoxification and hormone balance. We describe the first case of hepatotoxicity due to Estrium Whey in a 51-year old female with metastatic breast cancer with clinical, laboratory and histopathological changes.

Key words: Estrium Whey; Hepatotoxicity; Metastatic disease

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INTRODUCTION

Drug-induced hepatotoxicity is an important cause of hepatocellular injury and hepatic necrosis may range from asymptomatic elevations in transaminases to fulminant hepatic failure and death. The majorities of adverse liver reactions are idiosyncratic, and occur in most instances 5-90 d after the causative medication was last taken[1]. Non-conventional or alternative medical therapies are being used more frequently in Western society and should be considered as a possible cause of unexplained abnormal liver function tests[2].

Estrium Whey is an alternative nutritional support therapy for women. It's enhanced with specific nutrients including phytoestrogens, folate, antioxidants, and fiber to support healthy estrogen detoxification and hormone balance. Here, we report the first case of toxic hepatitis induced by Estrium Whey in a patient with metastatic breast cancer.

CASE REPORT

A 51-year old female with a history of metastatic breast cancer diagnosed in February 2010 came to our clinic with complaints of weakness, fatigue, jaundice and dark colored of urine beginning in the last 3 wk of estrium whey therapy which she had been taking for 3 mo as alternative treatment for her breast cancer (Table 1). She had not had contact with anyone with hepatitis. Her last chemotherapy was in July 2010, she had not used any tylenol including analgesics and anti-inflammatory drugs, in the previous 6 mo, she had no history of traveling.

Her vital signs on admission were stable. On physical examination she was conscious and icteric, the abdomen was no tender to palpation in right upper quadrant with no palpable organomegaly. She had no stigmata of

Table 1 Ingredients of Estrium Whey formula

Total fat	2 g
Saturated fat	0.5 g
Cholesterol	< 5 mg
Total carbohydrate	25 g
Dietary fiber	2 g
Sugars	19 g
Protein	15 g
Vitamin A (as retinyl palmitate)	1250 IU
Vitamin A (as beta-carotene)	2500 IU
Vitamin C (as ascorbic acid)	60 mg
Vitamin D (as cholecalciferol)	40 IU
Vitamin E (as d-alpha tocopheryl succinate)	300 IU
Thiamin (as thiamin HCl)	0.75 mg
Riboflavin	0.85 mg
Niacin (as niacinamide)	10 mg
Vitamin B6 (as pyridoxine HCl)	50 mg
Folate (as folic acid)	400 mcg
Vitamin B12 (as cyanocobalamin)	30 mcg
Biotin	150 mcg
Pantothenic acid (as D-calcium pantothenate)	5 mg
Calcium (as calcium citrate)	350 mg
Iron (as ferrous fumarate)	9 mg
Phosphorus (as dipotassium phpsphate)	260 mg
Iodine (as potassium iodide)	75 mcg
Magnesium (as citrate)	240 mg

Table 2 Liver function tests in the index patient

	On admission	Days after admission					References values
		2	3	4	5	7	
AST	1203	1600	1588	1693	2051	3050	< 43 U/L
ALT	625	635	613	616	652	173	< 45 U/L
BILI	5.7	7.5	9.1	9.6	10.4	14.1	< 1 mg/dL
AP	281	274	260	276	245	258	108 U/L

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BILI: Total bilirubin; AP: Alkaline phosphatase.

end stage liver disease. Cardiovascular and respiratory examination revealed no signs consistent with congestive heart failure or respiratory tract infection. Laboratory data at the time of admission showed: leucocytes 4300/mm^3, hematocrit 37.8%, hemoglobin 13.2 g/dL, platelet 247 000/mm^3, aspartate aminotransferase (AST) 1203 U/L (8-43), alanine aminotransferase (ALT) 625 U/L (7-45), alkaline phosphatase 281 U/L (41-108), total bilirubin 5.7 mg/dL, direct bilirubin 5.4 mg/dL, albumin 3.7 g/dL (Table 1). Urinalysis revealed positive bilirubin and positive urobilinogen. Viral serologic markers were as follows: Anti hepatitis A virus immunoglobulin M (IgM) negative, hepatitis B surface antigen negative, anti hepatitis B core IgM negative, anti hepatitis B surface indeterminate, anti hepatitis C virus negative, IgM and immunoglobulin G antibodies to cytomegalovirus, Epstein-Barr virus and herpes simplex virus negative, metals 63 AG negative. An ultrasonography at the time of admission showed multiple hypoechoic masses in both lobes of the liver likely due to metastatic disease, two echogenic nodules in the liver which are likely hemangiomas, spleen normal size with several small hypoechoic lesions that also could be metastatic disease, intrahepatic ducts,

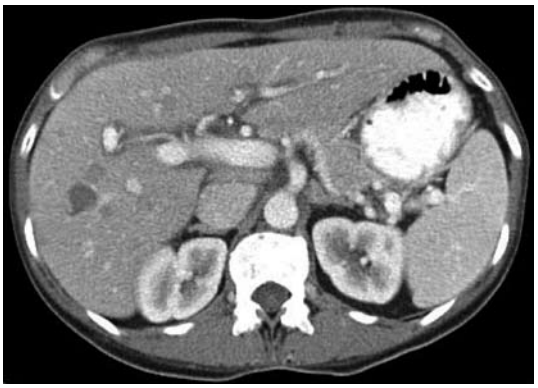

Figure 1 Computed tomography of the abdomen showing numerous masses in the liver and prominent lymph nodes.

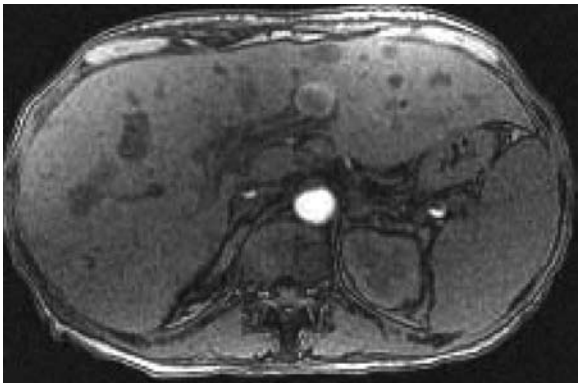

Figure 2 Magnetic resonance imaging of the abdomen with intravenous contrast showing metastatic disease.

and common ducts not dilated, gallbladder normal.

The patient was admitted to the hospital with the diagnosis of hepatitis, started on intravenous hydratation and subsequently ordered a computed tomography of the abdomen and pelvis, that revealed numerous masses within the liver, lesions within the spleen and prominent lymph nodes within the abdomen and pelvis consistent with metastatic disease (Figure 1), in suspicious of liver obstruction due to metastatic disease, we ordered a magnetic resonance imaging with and without contrast of the abdomen that showed multiple hepatic metastases scattered throughout the left and right lobes, 4 cavernous hemangiomas in the right hepatic lobe, mild periportal edema, patent portal and hepatic veins, prominent porta hepatics lymph nodes, multiple enlarged para-aortic and aortocaval lymph nodes, diffuse gallbladder wall thickening without luminal distention or pericholecystic fluid, multiple hypointense lesions throughout the spleen due to metastatic disease (Figure 2). Magnetic resonance cholangiopancreatography demonstrates no intrahepatic biliary dilatation, no common bile duct narrowing or dilatation.

Her hospital course was uneventful just for the ascending in her liver enzymes which now showed AST 2051 U/L, ALT 652 U/L, total bilirubin 10.4 mg/dL, international normalized ratio 1.1, alkaline phosphatase of 245 U/L (Table 2). The patient's jaundice and malaise

improved with supportive therapy, and she was discharged from the hospital.

Considering the clinical presentation, together with increased serum aminotransferase levels, absence of viral markers for hepatitis B, C and other hepatotropic viruses, evidence on images of no intrahepatic and extrahepatic dilatation, no enough metastatic disease to cause this rapidly progressive picture with very high AST, the final diagnosis was a toxic hepatitis induced by Estrium Whey.

DISCUSSION

Unconventional therapies have become more and more popular in Western society, and their use is not restricted to people and many of them have severe toxicities[3,4]. To define the risks of these preparations, the manufacturer should label all ingredients. Even when labeled, however, there are often significant discrepancies between the ingredients listed and the actual contents. Furthermore, these products are often adulterated with pharmaceuticals or contaminated with heavy metals[5].

The mechanism of Estrium Whey induced hepatotoxicity is not understood. The liver is the major site of metabolism of most drugs and is especially vulnerable to injury during biotransformation of these compounds. Since discontinuation of Estrium Whey was associated with resolution of symptoms and improvement of liver function abnormalities, drug induced hypersensitivity reaction seems very likely[6].

This patient was diagnosed with Estrium Whey induced toxic hepatitis. There was no history of alcohol or acetaminophen consumption, serological markers of viral hepatitis were negative and despite the liver metastasis that the patient had, the rapid increase in the liver enzymes and the histological findings were concordant with toxic hepatitis.

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CASE REPORT

Hepatic sarcoidosis complicating treatment-naive viral hepatitis

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Abstract

Hepatic sarcoidosis is usually asymptomatic but rarely leads to adverse liver-related outcome. Co-existence of viral hepatitis and hepatic sarcoidosis is a rare, but recognised phenomenon. Obtaining a balance between immune suppression and anti-viral therapy may be problematic. Immunosuppression in the presence of viral hepatitis can lead to rapid deterioration of liver disease. Similarly, anti-viral therapy may exacerbate granulomatous hepatitis. Here we present two cases of viral hepatitis co-existing with sarcoidosis that illustrate successful management strategies. In one, hepatitis B replication was suppressed with oral anti-viral therapy before commencing prednisolone. In the second, remission of hepatic sarcoidosis was achieved with prednisolone, before treating hepatitis C and obtaining a sustained virological response with pegylated interferon and ribavirin therapy.

Key words: Hepatic sarcoidosis; Chronic hepatitis C infection; Chronic hepatitis B infection; Immune suppression; Anti-viral therapy

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INTRODUCTION

Sarcoidosis is a progressive multi-organ disease of unknown aetiology, characterised histologically by the presence of non-caseating granulomas[1]. Clinical manifestations range from asymptomatic disease to multi organ failure. Hepatic involvement usually presents with abnormal liver biochemistry. Cirrhosis and liver failure are rare complications[2,3].

Co-existence of sarcoidosis and chronic viral hepatitis could accelerate liver fibrosis progression. Corticosteroids remain the mainstay of treatment for sarcoidosis. Treatment of hepatic sarcoidosis leads to symptomatic and biochemical improvement but may not necessarily impact disease progression[4]. On the other hand, immunosuppression with steroids could accelerate liver disease progression in patients with viral hepatitis. This phenomenon has been well documented with immune suppression during chemotherapy in patients with chronic hepatitis B virus (HBV)[5,6] and after liver transplantation.

Here, we report two cases of hepatic sarcoidosis complicating treatment-naive chronic HBV and hepatitis C

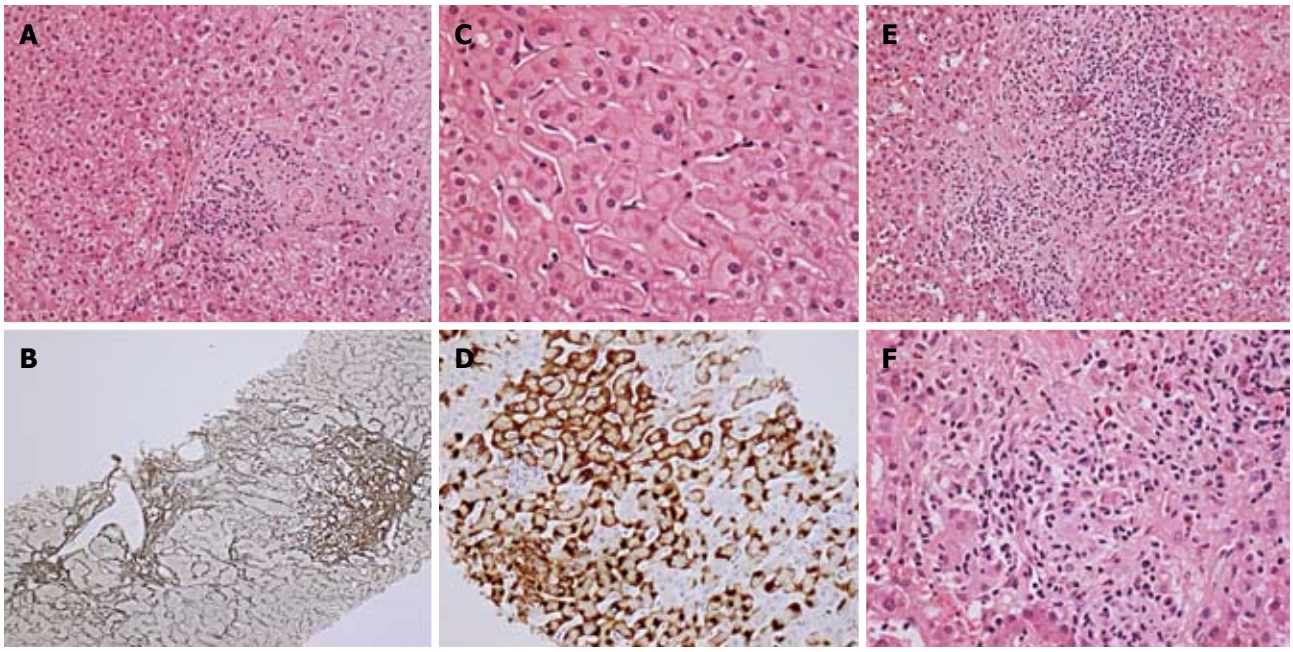

Figure 1 The histological features of hepatic sarcoidosis complicating chronic hepatitis B virus infection. A: Portal tract showing minimal portal inflammation attributable to hepatitis B virus [haematoxylin eosin (HE) staining ×20]; B: Portal fibrosis (reticulin ×40); C: Ground glass hepatocytes (HE ×40); D: Hepatitis B surface antigen immunostain showing accumulation in cytoplasm (×20); E: Granulomatous portal tract inflammation with duct irregularity (HE ×20); F: High power portal granulomatous inflammation (HE ×40).

virus (HCV).

CASE REPORT

Case 1

A 36 years old Ghanaian lady presented with abnormal liver biochemistry. Alanine transaminase (ALT) and alkaline phosphatase (ALP) were raised at 72 IU/L and 138 IU/L respectively (normal range ALT 0-54 IU/L; ALP 25-120 IU/L). Other than her country of origin, there were no risk factors for liver disease. A screen for chronic liver diseases demonstrated markers of chronic HBV carriage [hepatitis B surface (HBs) antigen positive, hepatitis B e (HBe) antigen negative, HBe antibody positive, HBV DNA 11 686 IU/L], but was otherwise unremarkable. Liver biopsy showed features of chronic HBV infection with moderate activity and moderate fibrosis. There were numerous ground glass hepatocytes and positive immunohistochemistry for HBs antigen (Figure 1A-D). Immunostaining for hepatitis B core antigen was negative implying low replicative activity. Additionally, there was non-caseating granulomatous portal inflammatory infiltrate (Figure 1E and F) noted. There was widespread mediastinal lymphadenopathy on computed tomography scanning, and the angiotensin converting enzyme (ACE) level was elevated (110 IU/L; normal range 12-68 IU/L). Other causes of granulomatous hepatitis were excluded.

Given moderate fibrosis on liver biopsy, lamivudine and adefovir were commenced. After 2 mo treatment, HBV replication was suppressed (HBV DNA < 100 IU/L), but abnormal liver biochemistry persisted. Prednisolone was therefore added to her regimen. Liver biochemistry and serum ACE level normalized (Figure 2), and HBV DNA remained undetectable though 24 mo follow-up. Current therapy consists of lamivudine, adefovir and prednisolone 10 mg.

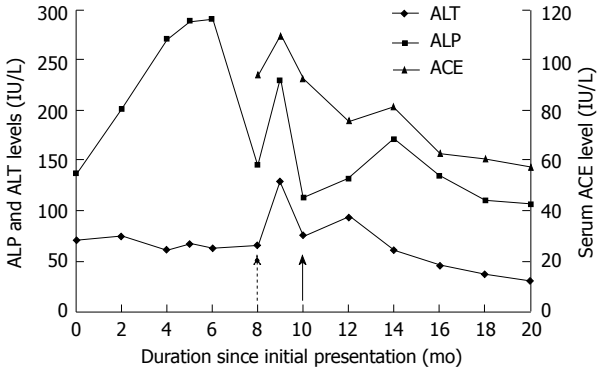

Figure 2 The changes in alanine transaminase, alkaline phosphatase and angiotensin converting enzyme levels in patient 1. The dotted arrow and the solid arrow mark the commencement of antiviral treatment and steroid treatment respectively. ALT: Alanine transaminase; ALP: Alkaline phosphatase; ACE: Angiotensin converting enzyme.

Case 2

A 37 years old man from Pakistan presented with a significantly elevated ALT level (532 IU/L, normal range 0-54 IU/L). Other than his country of origin, there were no risk factors for liver disease. A screen for chronic liver diseases demonstrated markers of chronic HCV carriage (HCV antibody positive, HCV RNA 4 450 000 IU/mL; genotype 3a), but was otherwise unremarkable. Liver bi-

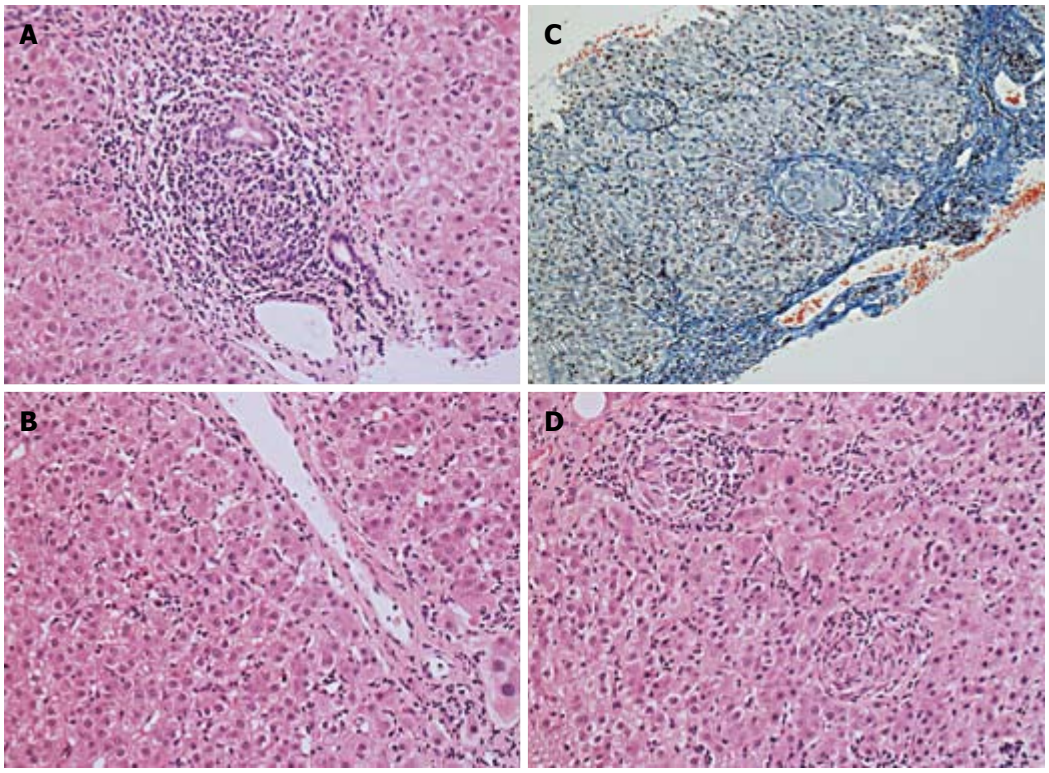

Figure 3 Histological features of hepatic sarcoidosis complicating chronic hepatitis C virus infection. A: Portal inflammation including lymphoid follicle and interface activity [haematoxylin eosin (HE) staining × 20]; B: Parenchymal inflammation and necroinflammation with acidophil bodies (HE ×20); C: Architectural stain showing parenchymal granulomatous inflammation and fibrosis (Chromotrope-Aniline Blue ×10); D: Parenchymal granulomatous hepatitis (HE ×20).

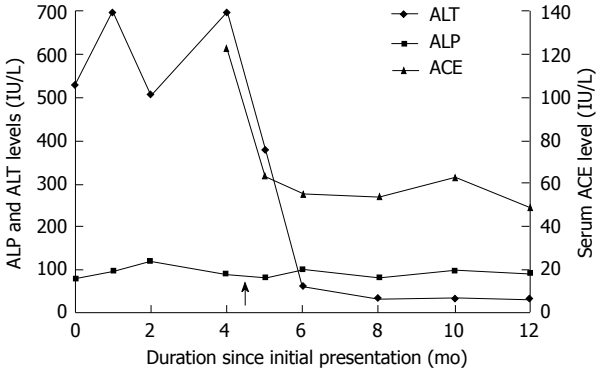

Figure 4 The changes in alanine transaminase, alkaline phosphatase and angiotensin converting enzyme levels in patient 2. The arrow marks the commencement of steroid treatment. ALT: Alanine transaminase; ALP: Alkaline phosphatase; ACE: Angiotensin converting enzyme.

opsy showed a moderately active portal and lobular hepatitis attributable to chronic HCV infection (Figure 3A and B) with moderate fibrosis. In addition, there were numerous small, well formed epithelioid granulomata seen throughout the lobule representing a granulomatous hepatitic component (Figure 3C and D). There was widespread mediastinal lymphadenopathy on computed tomography scanning, and the ACE level was elevated (124 IU/L). Other causes of granulomatous hepatitis were excluded with appropriate investigations. Steroid therapy was commenced and there was rapid normalisation of ALT and ACE levels (Figure 4). Following this, he received antiviral treatment with peginterferon alpha-2a 180 micrograms and ribavirin 400 mg twice daily for 24 wk. A sustained virologic response was achieved. He was maintained on prednisolone 10 mg throughout his antiviral treatment and thereafter.

DISCUSSION

Hepatic granulomas may be observed on liver biopsies from patients with hepatitis C[7,8], hepatitis B[9] and hepatitis A[10,11]. The incidence of hepatic granulomas in chronic HCV has been estimated at between 1%[7] and 10%[8]; in chronic HBV it is about 1.5%[9]. However, sarcoidosis complicating chronic viral hepatitis is rare. A number of case reports describe hepatic sarcoidosis in patients receiving antiviral treatment for HCV[12-18]. Here we report two cases of sarcoidosis complicating treatment-naïve chronic HBV and HCV. Sarcoidosis in untreated HBV is previously unreported.

Causes of hepatic granulomas include sarcoidosis, primary biliary cirrhosis, autoimmune hepatitis, drug-induced hepatotoxicity, lymphoma, viral hepatitis, tuberculosis, cytomegalovirus, leishmaniasis, toxoplasmosis, Q fever, fungal infections and antiviral treatment such as interferon, ribavirin and amantidine[8,19-21]. As for our patients, the diagnosis of hepatic sarcoidosis relied on demonstration of non-caseating granulomas and exclusion of other causes[22]. Whilst HCV and HBV may cause granulomatous hepatitis[7-9], the elevated serum ACE levels, extensive lymphadenopathy and steroid responsiveness supports a diagnosis of sarcoidosis in both cases.

The majority of patients with hepatic sarcoidosis are asymptomatic and the general consensus is to reserve treatment for patients with abnormal liver biochemistry[23]. Our cases fulfilled this criterion and deomonstrated normalization of liver tests with steriod therapy. For case 1,

abnormal liver biochemistry persisted despite HBV supression and then resolved with steroid therapy. For case 2, it was felt that the ALT level was much higher than what is usually seen in chronic HCV with moderate disease alone. This high ALT level and features of marked granulomatous hepatitis on liver biopsy led to initial therapy to be directed at sarcoidosis as this was considered to constitute the primary cause of liver injury. The ACE level dropped and liver biochemistry normalized with steroid therapy, even before the commencement of anti-viral therapy. Previous reports have documented a relapse of sarcoidosis with interferon treatment of HCV[15-18]. However, our patient (case 2) underwent successful therapy with pegylated interferon and ribavirin without such relapse.

In conclusion, hepatic sarcoidosis in combination with chronic viral hepatitis is uncommon. Our cases demonstrate that immune suppressive therapy in combination with appropriate timed antiviral therapy can be successful.

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CASE REPORT

Complications arising in simple and polycystic liver cysts

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Abstract

Liver cysts are common, affecting 5%-10% of the population. Most are asymptomatic, however 5% of patients develop symptoms, sometimes due to complications and will require intervention. There is no consensus on their management because complications are so uncommon. The aim of this study was to perform a collected review of how a series of complications were managed at our institutions. Six different patients presenting with rare complications of liver cysts were obtained from Hepatobiliary Units in the United Kingdom and The Netherlands. History and radiological imaging were obtained from case notes and computerised radiology. As a result, 1 patient admitted with inferior vena cava obstruction was managed by cyst aspiration and lanreotide; 1 patient with common bile duct obstruction was first managed by endoscopic retrograde cholangiopancreatography and stenting, followed by open fenestration; 1 patient with ruptured cysts and significant medical co-morbidities was managed by percutaneous drainage; 1 patient with portal vein occlusion and varices was managed by open liver resection; 1 patient with infected cysts was treated with intravenous antibiotics and is awaiting liver transplantation. The final patient with a simple liver cyst mimicking a hydatid was managed by open liver resection. In conclusion, complications of cystic liver disease are rare, and we have demonstrated in this series that both operative and non-operative strategies have defined roles in management. The mainstays of treatment are either aspiration/sclerotherapy or, alternatively laparoscopic fenestration. Medical management with somatostatin analogues is a potentially new and exciting treatment option but requires further study.

Key words: Liver; Cysts; Complications; Polycystic

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INTRODUCTION

Liver cysts can be single, multiple or diffuse, as in polycystic liver disease[1,2]. Simple cysts have a prevalence

of 5%-10% with a sharp rise in incidence with age[3]. Polycystic liver disease is an autosomal dominant condition caused by germline mutations of the *PRKCSH* and *SEC63* genes, which encode the β-subunit of glucosidase Ⅱ and Sec63 respectively[4]. Both proteins are components of the molecular machinery involved in the translocation, folding and quality control of newly synthesized glycoproteins in the endoplasmic reticulum[1]. Neoplastic cysts such as benign biliary cystadenomas and biliary cystadenocarcinomas are acquired, but the cause is unknown. Traumatic cysts are also acquired and result from bile leakage from an injured intrahepatic bile duct after trauma.

The majority of liver cysts are asymptomatic. They can occasionally be felt as a mass during physical examination. However most are found incidentally during abdominal imaging, especially on ultrasound. About 5% of patients are symptomatic and present with vague symptoms such as pain, nausea, early satiety, vomiting or heartburn[3]. Once found, there is a need to distinguish a simple cyst from other cystic lesions of the liver including hydatid cysts and cystic neoplasms. Previously reported complications of liver cysts include: intracystic haemorrhage[5], torsion[6], biliary fistula, rupture[7], infection[8], obstructive jaundice[9,10], malignancy[11], portal vein occlusion and varices[12], portal hypertension[13,14] and Budd-Chiari Syndrome[12].

Although complications of liver cysts are rare, they are often serious and sometimes life-threatening. There is no consensus on their management because complications are so uncommon. The aim of this study was to perform a collected review of how a series of complications were managed at our institutions.

CASE REPORT

Patient 1

A 75-year-old female patient presented with jaundice and abdominal pain. Liver function tests revealed obstructive jaundice and an ultrasound scan of the abdomen showed a 19 cm unilocular liver cyst and dilated intrahepatic biliary ducts. An endoscopic retrograde cholangiopancreatography (ERCP) was performed which confirmed blockage of the ductal system, a long stricture in the common bile duct and a stent was inserted to temporarily relieve the jaundice.

The computed tomography (CT) showed a large uncomplicated cyst occupying segments Ⅳ, Ⅴ, Ⅵ and Ⅷ. This was compressing the common bile duct causing obstruction with dilation of the intrahepatic bile ducts (Figure 1A). The magnetic resonance imaging (MRI) confirmed that the liver lesion was homogenous, suggestive of a simple liver cyst (Figure 2).

The patient underwent fenestration (deroofing) of the cyst occupying segments Ⅳ, Ⅴ, Ⅵ and Ⅷ and cholecystectomy. Three litres of clear fluid was drained, the roof of the cyst was excised and the cyst lining ablated with Argon Plasma Coagulation diathermy. She was reviewed in the out-patient clinic six weeks later where she reported no further pain, her jaundice had settled and the biliary stent was then removed at ERCP 1 wk later.

Patient 2

A 91-year-old female was admitted with right upper quadrant abdominal pain radiating to the right shoulder tip. She had a past medical history of simple liver cysts, ischaemic heart disease and chronic obstructive pulmonary disease with a poor exercise tolerance. On examination she had tender hepatomegaly. A CT scan showed free fluid in the abdomen and a large residual cyst in segments Ⅴ and Ⅵ (Figure 1B).

Due to her age, medical co-morbidity, and the fact that the cyst had already ruptured it was decided to opt for percutaneous drainage alone. Two drains were inserted; one into the cyst remnant, and another into the subphrenic collection. Apart from a right basal pneumonia, the patient made a full recovery and remains clinically well at 1 year.

Patient 3

An 82-year-old female was admitted with a three week history of indigestion and epigastric abdominal pain. Her past medical history was of hypertension, left ventricular heart failure and diverticular disease. On examination there was a large mass in the right iliac fossa. An ultrasound scan of the abdomen showed an 8 cm cyst arising from the liver and extending to the right iliac fossa. A MRI of the liver showed a complex exophytic lesion arising from segments Ⅴ and Ⅵ, with apparent internal membranes and fluid levels (Figure 3). Due to the indeterminate nature of the pathology and, despite negative hydatid serology, the patient underwent a liver resection, where a large cystic lesion, containing chocolate-like material was found, arising from segments Ⅴ and Ⅵ. This extended to the right iliac fossa, was attached to the colon and eroding into the small bowel. There were also multiple smaller liver cysts noted. The liver cyst was resected along with a loop of small bowel (Figure 4). Histology confirmed a haemorrhagic simple cyst. The patient made an uncomplicated recovery.

Patient 4

An 80-year-old patient presented with a two week history of abdominal pain and deranged liver function tests. An ultrasound of the abdomen showed a large liver cyst. A CT scan of the abdomen confirmed a large liver cyst occupying segments Ⅱ and Ⅲ compressing the portal vein leading to portal hypertension and splenic varices (Figure 1C and D).

The patient had a left hepatectomy. A large simple cyst was found causing portal vein obstruction with splenic varices. He had no post-operative complications.

Patient 5

A 44-year-old female with autosomal dominant polycystic liver disease was admitted because of massive oedema of her lower extremities extending to her abdominal wall. Her weight had increased by 15 kg. These

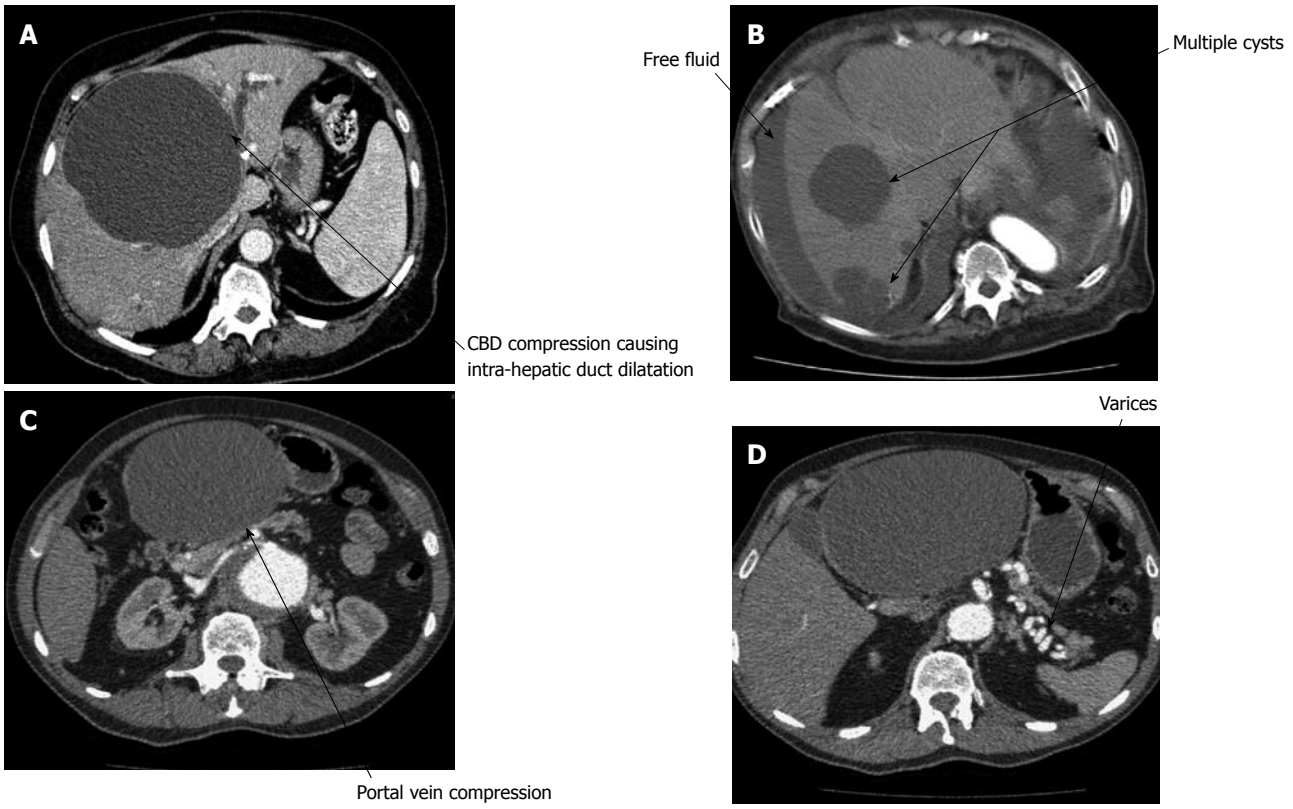

Figure 1 Computed tomography. A: The liver cyst causing common bile duct compression and dilation of the intrahepatic bile ducts; B: Multiple liver cysts with free fluid around the liver; C: The cyst compressing the portal vein; D: The splenic varices from the resulting portal hypertension.

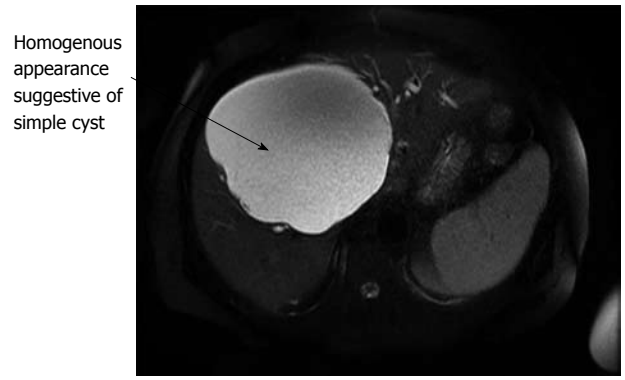

Figure 2 A cross-sectional magnetic resonance imaging scan confirming the homogenous appearance of the cysts suggestive of a simple liver cyst.

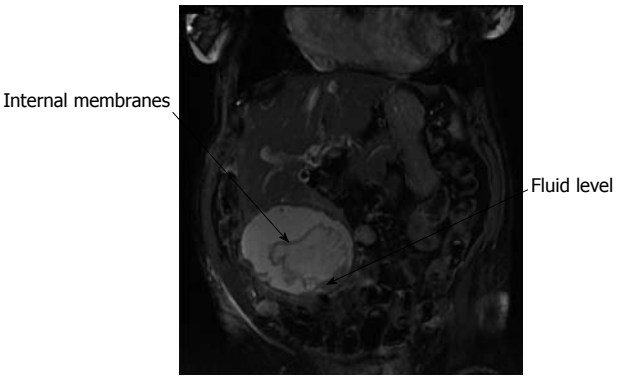

Figure 3 Coronal magnetic resonance image showing the liver lesion with apparent internal membranes extending from segments Ⅴ and Ⅵ of the liver to the right iliac fossa.

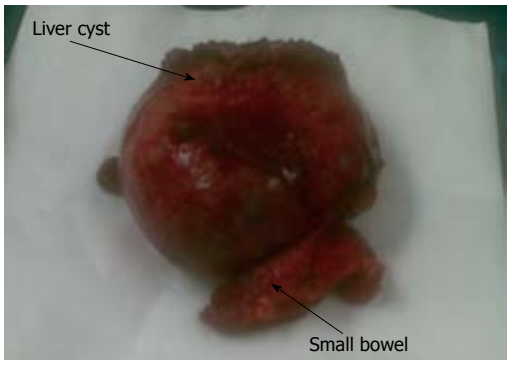

Figure 4 The resected liver cyst of patient 3 with attached small bowel.

symptoms were precipitated by laparoscopic fenestration of multiple liver cysts. CT scanning revealed the presence of ascites in the lower abdomen, while the upper abdomen was completely occupied by the polycystic liver (Figure 5). CT showed compression of the inferior vena cava. In order to relieve the caval pressure, 3 strategically located cysts were aspirated, the ascites was drained and diuretics and somatostatin analogues were started. She subsequently made a good recovery.

Patient 6

A 47-year-old female with autosomal dominant polycystic

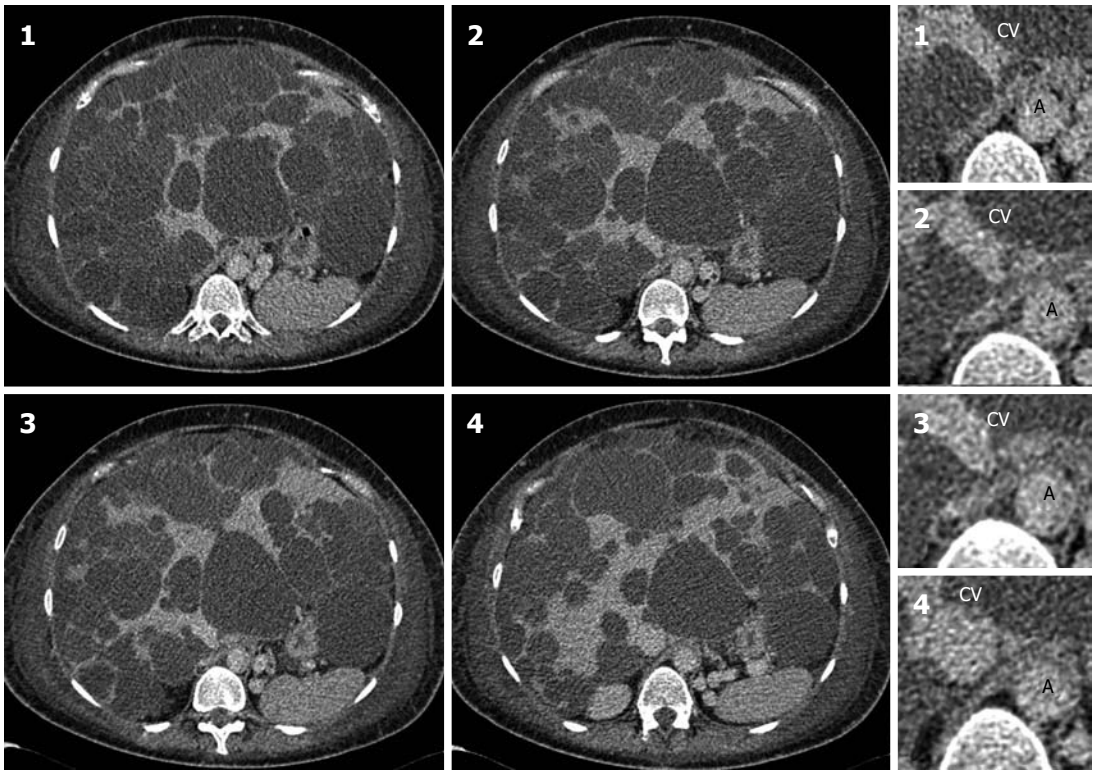

Figure 5 A transverse section of the abdomen on computed tomography scanning. The left 4 panels represent cranio-caudal sections of the abdomen while the right 4 panels represent magnifications. Panel 4 shows the normal inferior vena cava (CV) and aorta (A), but the CV becomes compressed as can be seen in panel 1-3.

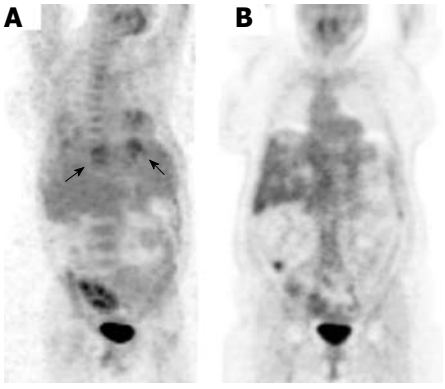

Figure 6 A positron emission tomography-computed tomography scan during the infection (A) and after treatment (B). Picture A shows appearances consistent with multiple infectious cysts, with a medium intense, circular fluorodeoxyglucose-accumulation in the middle of the right liver.

liver and kidney disease, was admitted because of pneumonia and multiple liver cyst infection with *Klebsiella pneumoniae*, diagnosed by blood culture. The liver was grossly enlarged because of numerous cysts. The liver volume was 4.5 L. The infection was successfully treated with ciprofloxacin. Two years later, the liver cysts recurred and blood culture grew *Klebsiella pneumoniae*, which was treated with oral ciprofloxacin 1 g/d for one year. One year later, she was again admitted because of recurrent infectious liver cysts, this time with an *Escherichia coli* (*E. coli*) infection. This was treated with cefuroxime 3 × 1.5 g *iv* and a single dose of gentamicin. The positron emission tomography CT (PET-CT) scan showed fluorodeoxyglucose (FDG) accumulation at the rim of the cyst. After antibiotic treatment, these FDG-accumulations disappeared (Figure 6). She subsequently developed multiple recurrences of infected liver cysts, most recently with an extended spectrum beta-lactamase-positive *E. coli*.

Because of the multiple recurrences, the resistant organisms and the fact that the infected liver cysts were spread throughout the liver, the patient is currently waiting for a liver transplantation.

DISCUSSION

Therapeutic intervention is warranted in the management of symptomatic liver cysts or when complications occur. For symptomatic cases, procedures available include: percutaneous aspiration with ethanol sclerotherapy, laparoscopic or open cyst fenestration (deroofing), hepatic resection and liver transplantation. The somatostatin analogue, lanreotide, reduces the volume of polycystic livers but has a modest clinical effect[15]. Complications of cystic liver disease are rare, and we have demonstrated in this series that both operative and non-operative strategies have defined roles in management.

Biliary obstruction can initially be relieved with ERCP and stent insertion but definitive treatment is required. In this series, definitive treatment was with liver cyst fenestration. Laparoscopic fenestration has largely superseded the open approach for symptomatic liver cysts. This treatment is particularly suited to solitary liver cysts, par-

ticularly if more anteriorly located. With careful selection, good operative technique and, in a high-volume centre, good results can be obtained. A recent series of 51 patients reported complete relief of symptoms at a median follow-up of 13 mo, nine minor perioperative complications and a median hospital stay of 2 d[16]. Symptomatic relief and a median volume reduction of 12.5% has been achieved in patients with polycystic liver disease[17]. Percutaneous aspiration with ethanol sclerotherapy would be an acceptable alternative strategy[18], as although the cysts will not disappear, they will reduce in size and this may be sufficient to relieve the extrinsic compression by the cyst on the bile duct.

Liver cyst rupture was managed conservatively with percutaneous drainage in the second patient in this series, due to the patient's multiple medical co-morbidities and age. Although simple percutaneous drainage of liver cysts is almost inevitably associated with recurrence[19], it was used in this case because the cyst had already ruptured and surgery was not an appropriate option for this patient. Sclerotherapy was not performed because the cyst had already ruptured.

Diagnostic uncertainty often results in surgical resection, in order not to miss a neoplastic condition, such as cystadenoma or cystadenocarcinoma or where the cyst has features of hydatid disease. The latter consideration, coupled with symptoms, led to the decision to undertake open resection of the cyst, with an adjacent segment of small bowel in patient 3. The bowel was intimately adhered to the cyst wall, presumably as a result of a cyst rupture, which the bowel had contained. Hydatid serology was negative in this case but a negative result is not completely reliable and immunodiagnosis plays a minor role in the definitive diagnosis of hydatid disease[20].

Portal vein occlusion, associated with splenic varices occurred as a complication in patient 4. Although segment Ⅱ and Ⅲ liver resection is now commonly performed laparoscopically, the presence of portal hypertension and varices indicated that the patient was at high risk of haemorrhage so open resection of the cyst was performed. Experience with liver resection for cystic liver disease is limited; the largest series published to date reported on 124 patients with polycystic liver disease undergoing partial hepatectomy with cyst fenestration, over a 21-year period[21]. Although good symptom relief were obtained, this was at a cost of a 63% in-hospital complication rate and a 3% mortality rate. This operation is technically demanding, as the polycystic liver is large and rigid with decreased mobility and reduced access to vascular inflow and outflow. Hepatic transection is complicated by displacement of hepatic veins and bile ducts from their normal anatomical positions. Liver resection is not, therefore, a first-line treatment for liver cysts but has a particular role when doubt as to the nature of the cyst exists[22].

The fifth patient presented with ascites and obstruction of the inferior vena cava. This was treated by a combination of cyst aspiration, diuretics and somatostatin analogues. The rationale for somatostatin analogue therapy is its inhibitory effect on cholangiocyte proliferation and cyst fluid secretion[23]. The clinical effect of this has recently been evaluated in a randomised double-blind, placebo-controlled trial with the somatostatin analogue, lanreotide in patients with polycystic liver disease[15]. Although an effect on the natural course of the disease was confirmed, this only amounted to a 2.9% reduction of liver volume. Further clinical trials are required before this treatment can be recommended for adoption into practice.

Finally, we demonstrate the utility of PET-CT imaging in demonstrating complete resolution of infected liver cysts with appropriate antibiotic therapy. Multiple proven recurrences have led to this patient with polycystic liver disease being referred for liver transplantation. Liver transplantation remains the only curative option for the minority of patients with severe polycystic liver disease who have disabling symptoms and poor quality of life. Recent results of liver transplantation are encouraging. In a large cohort of 58 patients with isolated polycystic liver disease the 5-year patient survival was 92% and in the cohort with 121 patients with polycystic kidney and liver disease this survival rate was 89%[24]. The collective experience on 218 patients from the European Liver Transplant Registry indicated a 5-year survival of 80%[25].

The current series has demonstrated several rare complications of simple liver cysts and polycystic liver disease. As these are rare, there are no consensus treatment strategies, but we highlight the role of tailored individual treatment, taking into account, cyst site and size, possibility of malignancy, medical co-morbidities and technical feasibility. We have shown that a role for liver resection remains, especially when there is diagnostic uncertainty. The mainstays of treatment are either aspiration/sclerotherapy or, alternatively laparoscopic fenestration. Medical management with somatostatin analogues is a potentially new and exciting treatment option but requires further study.

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CASE REPORT

Atrial embolism caused by portal vein embolization: Treatment by percutaneous withdrawal and stenting

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Abstract

Hepatectomy remains the only curative treatment for many primary and secondary liver cancers. Portal vein embolization (PVE) has been used to increase the volume of the future liver remnant and thus lower the risk of small-for-size syndrome and postoperative liver failure. This technique has proven its safety, with a low post-procedure morbidity rate. Here, we describe a very rare case in which a young patient suffered a glue embolism to the right atrial cavity following PVE in preparation for a major hepatectomy for colorectal metastasis. The foreign body was withdrawn from the heart with a femoral, percutaneous device and trapped against the wall of the femoral vein with a self-expanding metal stent. Our report shows that this previously unknown complication of PVE can be resolved without recourse to sternotomy and open heart surgery.

Key words: Liver metastasis; Percutaneous endovascular intervention; Portal vein; Embolization; Glue; Complications; Stenting

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INTRODUCTION

Portal vein embolization (PVE) is used to induce liver hypertrophy prior to a major hepatectomy and thus to lower the risk of postoperative failure associated with an insufficient future liver remnant (FLR) volume. Ever since Makuuchi *et al*[1] described the first PVE in 1984, this type of preoperative management has proven itself to be effective and has been adopted worldwide. In a meta-analysis of 37 studies, Abulkhir *et al*[2] reported post-PVE percentage increases in the FLR ranging from 8% to 27%. This enables many supposedly unresecable patients to become resecable after PVE.

Despite the good overall safety of PVE as also em-

phasized in the meta-analysis[2], complications do still occur. These notably include liver hematoma, liver abscesses, cholangitis and also problems related to overflow of embolization glue into the non-embolized liver. Indeed, main or left portal branch thrombosis have been described as major complications resulting from this type of foreign body embolism[2].

The present clinical case report highlights atrial glue embolism as a complication of PVE and describes the management of this rare event. The frequency of post-PVE glue embolism has not been reported but the physician should bear in mind that percutaneous treatment is possible.

CASE REPORT

A 31-year-old man was referred to our unit for further evaluation of a left colon cancer with synchronous bilobar hepatic metastases. After colectomy, the patient was proposed a two stage hepatectomy. The first stage consisted of the resection of four metastases in the left lateral section. The patient was then scheduled for PVE in order to increase the left lateral section volume and a right extended hepatectomy. The procedure was complicated by the patient's portal anatomy. The radiologist was eventually able to access the right portal branch by puncturing the left branch and then injecting a 1:1 mixture of n-butyl-2-cyanoacrylate (NBC, Histoacryl®, B Braun, France) and Lipiodol® (Guerbet, France). Embolization of the right posterior branch was problem-free. At the beginning of the embolization procedure for the right anterior branch, the entire embolization mixture passed through a portosystemic shunt that had not been seen in the previous portography. The glue passed through a hepatic vein into the vena cava and then into the right atrial cavity (Figure 1). An emergency cardiac echoendoscopy examination confirmed the presence of a mobile foreign body in the right atrial cavity, with a high risk of pulmonary embolism. After a multidisciplinary staff meeting, we ruled out surgical management because of the risk of potential morbidity and decided to attempt percutaneous treatment.

An 8F basket catheter was introduced into the right femoral vein and pushed through the vena cava into the right atrial cavity. The foreign body was captured (Figure 2) and pulled through the vena cava and the right femoral vein. Because of the risk of fragmentation of the foreign body during extraction, a self-expanding Nitinol stent (Smart®, Cordis, Issy les-Moulineaux, France) 14 mm in diameter and 6 cm in length waDr. a second puncture situated a few centimeters below the first one. The two first centimeters at the proximal edge of the stent were opened before the basket catheter opened, in order to release the foreign body without embolic event. Then the stent was fully implanted and was covering the glue and the embolic material that was pushed against the vein wall. At the end of the procedure, final angiogram showed blood flow into the prosthesis with no signs of

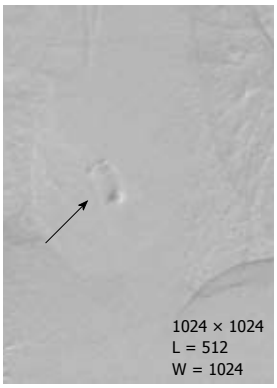

Figure 1 Portal vein embolization material embolism in the right atrial cavity (arrow).

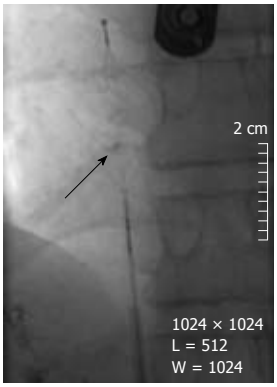

Figure 2 Material withdrawal through the vena cava with a basket catheter (arrow).

thrombosis (Figure 3). Cardiac echoendoscopy confirmed the complete removal of the foreign body. The endovascular intervention was complicated by a groin hematoma that required a compression bandage but resolved spontaneously without any transfusion and increase of hospitalization stay. There was no edema of the leg or other vascular complications. The patient was discharged on day 3 with an anticoagulant treatment (enoxaparin sodium, 4000 IU/d) for a month because of the risk of cancer-related thrombosis.

Given that the PVE had not been completed, the patient could not be operated on; extended hepatectomy was considered to be unsafe in the absence of sufficient hypertrophy of the FLR. He received palliative chemotherapy and died 29 mo later from cerebral metastasis.

DISCUSSION

In this case report, NBC was used to embolize the right portal vein. Although this substance is used routinely for PVE in safe conditions[3], it is friable and was reportedly responsible for a pulmonary embolism following gastric variceal obliteration in cirrhotic patients[4]. There are two possible mechanisms for the migration of PVE material. The first relates to a spontaneous, intrahepatic portosystemic venous shunt that, although rare, has been already

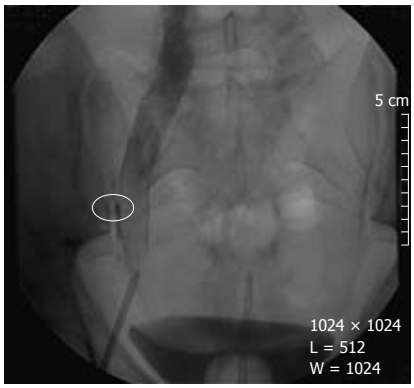

Figure 3 Femoral stent placement. The stent pressed the portal vein embolization material against the wall of the femoral vein (circle). Note the good venous flow at the end of the procedure.

described a few times[5]. The second possibility is the creation of an iatrogenic portosystemic venous shunt by high pressure injection of the NBC; however, this entity has not been yet described in the literature.

In the present case, our decision to use a percutaneous technique to retrieve the migrated material was prompted by two factors. Firstly, sternotomy and open heart surgery for foreign body extraction had a high risk of morbidity. Secondly, a cardiopulmonary bypass was ruled out because the required doses of anticoagulant and the multiple attempts to access the left portal branch for PVE would have been associated with a high risk of liver subcapsular hematoma.

Extraction of material from the right cardiac cavity using a basket catheter has already been described. This technique was used for extraction of thrombi in patients contraindicated for surgery, with the placement of an inferior vena cava filter above the thrombus[6]. Lastly, given the high risk of fragmenting the PVE material during its extraction from the femoral vein, a femoral stent was used to trap the material against the vein wall and enlarge the vein. Anticoagulation was used to reduce the risk of venous thrombosis that could be potentialized by cancer (Figure 3). Venous stenting has already been reported and has proven its efficacy[7].

In conclusion, we have described a very rare case of a PVE material migration to the right atrial cavity. The embolism was successfully treated by withdrawal with a basket catheter and then femoral venous stenting. The mechanism of portosystemic venous shunting in this case was not clear but our report should prompt the physician to examine the patient's portal anatomy even more carefully prior to injection of a PVE product. This previously unknown complication of PVE can be managed percutaneously in order to avoid open heart cardiac surgery.

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CASE REPORT

Liver metastasis of endometrial stromal sarcoma

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Abstract

Resection of liver metastases from gynaecological tumours is uncommon. Endometrial stromal sarcomas (ESS) are low incidence gynecological tumours which can originate in previous sites of endometriosis or following metaplasia of the pelvic peritoneal wall, and which are exceptionally associated with liver metastasis. We present a 68-year-old woman with a ESS and metachronic liver metastasis treated by liver resection. There is very little literature on clinical management about liver metastasis from ESS, but extrapolating the data obtained with liver metastasis from sarcomas and uterine tumours, we should recommend resection as this is considered a resectable extrauterine metastasis. In cases with more sites of extrauterine disease, liver resection should also be performed if the other sites are resectable.

Key words: Sarcoma; Stromal; Endometrial; Liver; Metastasis; Review

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INTRODUCTION

Resection of liver metastases (LM) caused by non-colorectal, non-endocrine tumours has progressed from being an exceptional indication to being admitted as the treatment of choice for certain types of neoplasias[1]. Resection of LM from gynaecological tumours is uncommon[1,2]. Endometrial stromal sarcomas (ESS) are low incidence tumours which can originate in previous sites of endometriosis or following metaplasia of the pelvic peritoneal wall, and which are exceptionally associated with liver metastasis[2-4]. The reported cases of resection of ESS LM are very rare[2].

CASE REPORT

A 68-year-old woman, with a history of hysterectomy with bilateral oophorectomy because of multiple myomas performed in a hospital in France in 1981. In 1996, the patient was operated in our hospital because of multiple peritoneal implants diagnosed as stage 4B solid-cystic endometrial stroma affecting the peritoneal wall. The patient received post-operative chemotherapy (doxorubicin, iphosphamide and docetaxel). In 2003, an abdominal computed tomography (CT) follow-up detected a 3-cm nodule on the jejunal loop and a 10 cm. Mass which involved the sigmoid colon, for which sigmoid-

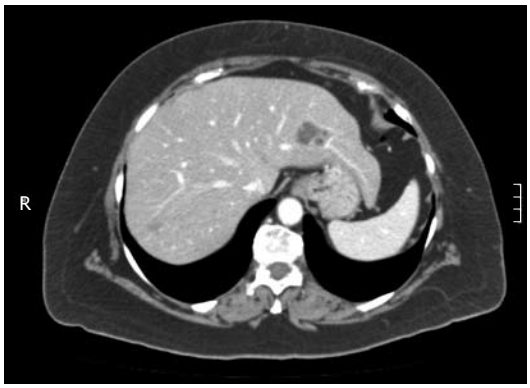

Figure 1 Solid-cystic lesion in left lateral sector of the liver.

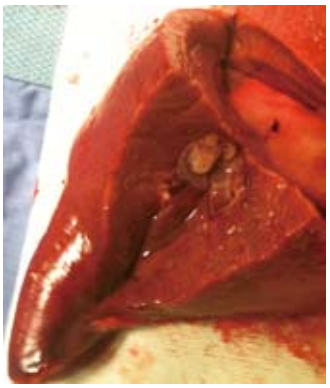

Figure 2 Surgical specimen: Solid cystic-lesion (liver metastasis of endometrial stromal sarcomas).

ectomy and intestinal resection were performed; ESS metastasis was also diagnosed. From 2003 to present the patient has been treated with letrozole; periodic radiological follow-up was performed. On the last abdominal CT and magnetic resonance imaging (MRI) performed (June 2010) we observed a 26-mm lesion located in the left lateral area of the liver with poorly defined borders, solid and cystic areas, with heterogeneous contrast uptake (Figure 1). In addition, we observed two previously known hemangiomas in the right liver lobe. There were no radiological data on intra-abdominal recurrence. Positron emission tomography (PET) was performed which did not reveal uptake in the lesion described. Given the onset of a new liver lesion with malignant appearance, in spite of the negativity of PET, we decided to operate and perform a left lateral sectionectomy via subcostal laparotomy. We previously assessed the abdominal cavity confirming that there were no other sites of tumour recurrence. Post-operative clinical course was normal and the patient was discharged on day 3. Macroscopically, the lesion was located 2.5 cm from the surgical margin and presented a cystic appearance with a solid area of yellowish colour and solid appearance (Figure 2). Microscopically, we observed in the liver parenchyma, with a predominantly bile periductal pattern, multiple nodules from tumour proliferation with a mesenchymal appearance similar to the endometrial stroma, with cells from ovoid or elongated nuclei which presented slight atypical cytology. The mitotic index was 8 mitosis figures in 10 enlarged fields (Figure 3). We observed marked immunostaining to oestrogen and progesterone receptors, alpha-actin and CD10, and negativity to desmin and c-kit. All these findings were highly suggestive with LM from a low-grade ESS (Figure 3), and very similar to those found in the two previous metastases. Treatment was started with megestrol acetate but this had to be discontinued because a deep vein thrombosis occurred. During 6 mo follow-up there were no signs of recurrence.

DISCUSSION

Uterine sarcomas comprise less than 1% of gynaecological cancers and 2% to 5% of all uterine cancers[4,5]. There are various subtypes of uterine sarcoma, including ESS which are sarcomas arising in the endometrial stroma; they are usually low-grade when present in premenopausal patients and high-grade in menopausal patients[4,5]. Cases of ESS account for 10%-15% of all uterine sarcomas and 0.2% of total uterine neoplasias. They are made up of cells similar to the endometrial stroma in their proliferative phase, and have the potential to multiply and metastasise[4,5]. There are different macroscopic appearances of the ESS: single nodule, solid-cystic mass, poorly differentiated lesion or multilocular cystic lesions[4]. Recurrence of ESS despite radical surgery is commonplace and occurs in 36% to 56% of patients with early tumours[4,5]. Indolent growth of recurrences leads to performing repeat surgery and repetitive cytoreduction surgery[4].

The existence of extrauterine disease in ESS occurs in 45% of patients but very rarely in the liver[6]. The number of ESS LM published is exceptionally low; during an intense literature search we only found one other case of resection[2]. Resection of metastasis at a localised distance from the lung or heart is accepted as valid treatment[5], but because of the reduced number of patients with ESS LM there is no scientific validation of the therapy to be used. The cancer clinical practice guide of the National Comprehensive Cancer Network - Version 1.2011 considers that the treatment of choice for isolated disseminated disease from uterine sarcomas is surgical resection with post-operative hormone therapy as we performed in our case[7].

The resection of LM from gynaecological tumours is still rare. The larger multicentre series for non-colorectal, non-endocrine LM includes 43 patients with LM from uterine tumours without specifying the histological lineage, obtaining a 5-year survival rate of 35 % with a median of 32 mo. In the same series, patients with LM from sarcoma from different sites had 5-year survival of 31% with a median of 32 mo[1]. In a series on 66 cases of LM from sarcoma from different sites, 3 were localised in the uterus without specifying the histological lineage[8]. In this series, in addition, it is also stated that sarcoma LM are not usually sensitive to chemotherapy or chemoembolisa-

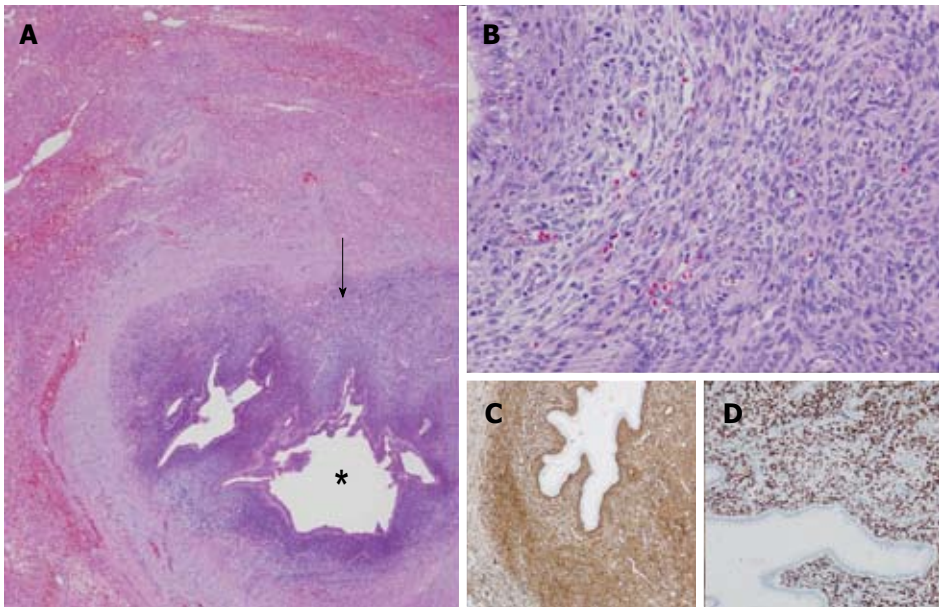

Figure 3 Microscopy. A: Low-magnification microphotography of the metastasis. Periductal infiltration of the neoplasia (arrow). The area of the asterisk corresponds to the lumen of a large bile duct; B: A high magnification shows mesenchymal neoplastic proliferation with little atypia similar to the endometrial stroma; C, D: Positive immunostaining of the neoplasia compared to alpha-actin (C) and oestrogen receptors (D).

tion, for which reason surgery with a free margin should be considered the best therapeutic option[8].

ESS may occur in extrauterine locations in the absence of a primary lesion which makes diagnosis markedly difficult[2,3]. Extrauterine ESS originate in previous sites of endometriosis or following metaplasia of the pelvic peritoneal wall[2,3]. Therefore, in the event of a liver lesion with ESS histology with no primary uterine lesion we should make a distinction between malignant liver endometriosis, liver primary extrauterine ESS, or undiagnosed liver metastasis from an ESS[2]. For our patient, the previous history made the diagnosis of ESS LM easier.

The number of published cases of liver endometriosis is also very low as there are only 12 cases published to 2009, with a malignancy rate of 25%[9,10]. The implantation mechanism of endometriosis in the liver is unknown[2,10], the proximity of liver endometriosis communicated to the falciform ligament suggests that the most probable route is vascular through the falciform ligament vessels or the umbilical vein[2,10].

The CT image of ESS LM does not have specific characteristics. For our patient, it was a solid-cystic mass just as the primary ESS-an uncommon fact in LM caused by tumours in other organs. This finding, in patients with no previous history of ESS, may lead to an erroneous diagnosis of malignant or complicated cystic tumour[2,9,10]. The PET has shown a sensitivity of 80% to detect extrapelvic metastasis of uterine sarcomas[11], but the experience for ESS is low. The PET was negative in our patient.

The prognosis of ESS is correlated with the mitotic index, the absence of invasion of the myometrium and the presence of extrauterine disease[2,4,6]. Overall survival at 5 years for the ESS is 65%, but it decreases to 32% in those with extrauterine disease. No data are available on survival following resection of ESS LM because of its exceptional nature.

In conclusion, ESS LM are exceptionally rare. There is very little literature on clinical management but we believe that by extrapolating the data obtained with LM from sarcomas and uterine tumours and following the recommendations of the NCCN clinical guide we should recommend resection as this is considered a resectable extrauterine metastasis. In cases with more sites of extrauterine disease, resection of LM should also be performed if the other sites are resectable.

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CASE REPORT

Life-threatening hemorrhage after liver radiofrequency ablation successfully controlled by transarterial embolization

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Abstract

A 59-year-old man underwent liver radiofrequency ablation under laparotomy for recurrent hepatic carcinoma located in the caudate lobe, however, near-fatal bleeding occurred 1 wk after the operation. The intraoperative ultrasound study during laparotomy revealed left hepatic artery pseudoaneurysm. Suture and packing with ribbon gauze was used to obtain hemostasis. A secondary hemorrhage followed 11 h later and hepatic angiography was performed immediately. Bleeding from the pseudoaneurysm in a branch of the left hepatic artery was found and the artery branch was embolized with coils. Other than slight bile leakage, post-embolization continued satisfactorily. Bleeding did not reoccur. The follow up visit 1 mo later found the pseudoaneurysm disappearing and no tumor recurrence.

Key words: Hepatocellular carcinoma; Radiofrequency ablation; Complication; Hepatic angiography; Embolization

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Wu XY, Shi XL, Zhou JX, Qiu YD, Zhou T, Han B, Ding YT. Life-threatening hemorrhage after liver radiofrequency ablation successfully controlled by transarterial embolization. *World J Hepatol* 2012; 4(12): 419-421 Available from: URL: http://www.wjgnet.com/1948-5182/full/v4/i12/419.htm DOI: http://dx.doi.org/10.4254/wjh.v4.i12.419

INTRODUCTION

Liver radiofrequency (RF) ablation has been accepted as a safe and useful therapeutic option for treatment of both primary and secondary hepatic neoplasms[1]. The most common complication is bile leakage which necessitates abdominal drainage in about 3.3% of procedures[2]. The hemorrhagic complication is very rare, but can be catastrophic, and lead to death[3]. We encountered a case with massive bleeding following liver RF ablation treatment of a hepatic artery rupture. This report describes our experience in dealing with this type of complication.

CASE REPORT

A 59-year-old man who had received liver RF ablation 11 times previously was admitted to receive further liver RF ablation for treatment of a recurrent hepatic carcinoma. Liver RF ablation was performed in Jan 2011. He had hepatitis B-related liver cirrhosis. Child-Pugh score was class A. Coagulability was within the normal range (international normalized ratio, 1.06). At the time of liver RF ablation, 5 hepatic lesions were found. The α-fetoprotein level was elevated to 133 ng/mL. First we ablated 4 peripheral lesions, and the lesion located in caudate lobe was ablated finally (Figure 1). Liver RF ablation was done under laparotomy. Cool-tip RF electrodes (Radionics, Burlington, MA, United States) were placed in three different sites of the tumor. Then RF energy was applied for 15 min to each site. An abdominal

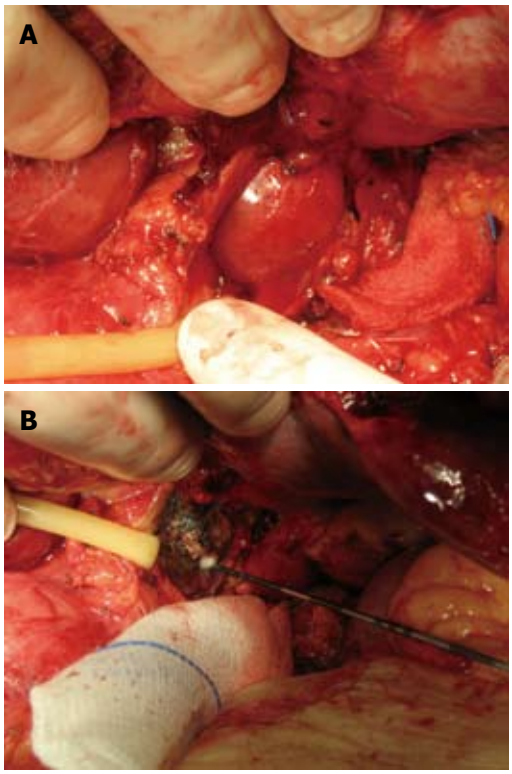

Figure 1 The exposure and radiofrequency ablation of the lesion in the caudate lobe under laparotomy. A: The tumor found in the caudate lobe. The arrow indicates the lesion; B: The largest tumor in the Caudate lobe measuring 4 cm was treated with radiofrequency (RF) ablation, with RF electrodes were placed at the tumor site.

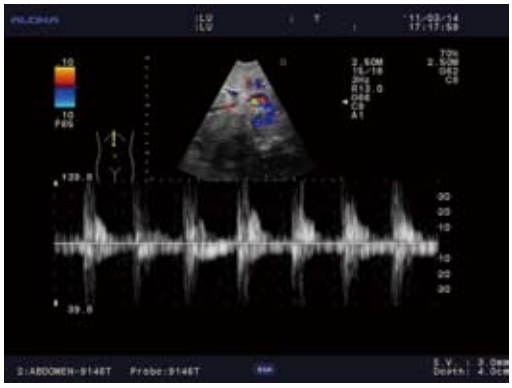

Figure 2 Introperative ultrosound study showed a hepatic artery pseudoaneurysm within the hematoma.

tube was placed under the left hepatic lobe. Near-fatal bleeding developed 1 wk after RF ablation.

Vital signs were unstable; the patient presented with acute hemorrhagic shock immediately. The hemoglobin level decreased by 6 g/dL and the patient was transferred immediately to the operating room. Explorative laparotomy was carried out. More than 4000 mL blood was lost in the abdominal cavity, active bleeding from the caudate lobe was identified. A hepatic artery pseudoaneurysm measuring 15 mm maximum in diameter was observed in the hematoma using intra-operative ultrasound (Figure 2). The bleeding site was first sutured

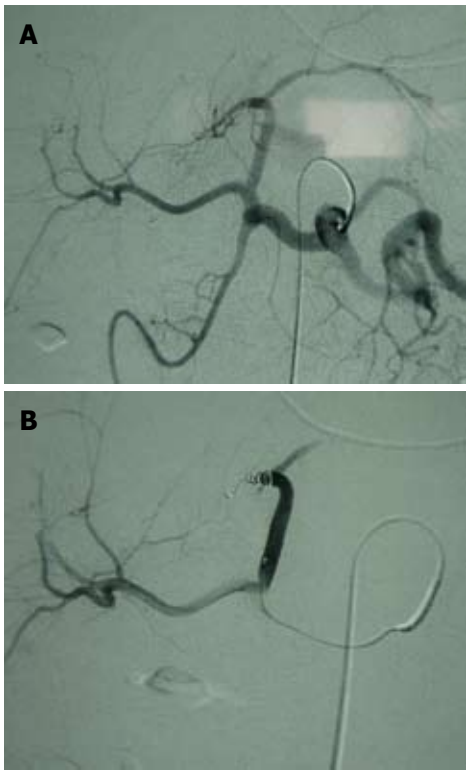

Figure 3 Hepatic angiography before and after coil embolization. A: Hepatic angiography revealed a pseudoaneurysm in the lower left hepatic artery; B; Hepatic angiography immediately after coil embolization. Coils were placed using a 2.1 F microcatheter into and proximal to the pseudoaneurysm. The pseudoaneurysm was then excluded.

and then packed with ribbon gauze for hemostasis, vital signs returned to normal and the patient was moved to intensive care. Eleven hours later, fresh blood appeared in the abdominal tube, the drainage was more than 100 mL/h, heart rate increased subsequently, the patient was moved to the angiographic suite.

Angiography revealed bleeding from the pseudoaneurysm in a branch of the left hepatic artery (Figure 3A). A microcatheter (2.0 Fr, Progreat; Terumo Corp, Tokyo) was advanced into the pseudoaneurysm through a 5-Fr catheter. Subsequently, five microcoils (Tornado Embolization Microcoils; Cook Medical Inc, Bloomington, IN) were deployed into and proximal to the pseudoaneurysm through the left hepatic artery. Immediately after coil embolization, the pseudoaneurysm was excluded successfully (Figure 3B). Other than a little bile leakage, the patient recovered smoothly after left hepatic arterial embolization. A contrast enhanced computed tomography (CT) study acquired 1 month later revealed that the pseudoaneurysm had disappeared. No tumor enhancement was apparent in the ablated site. The coils used for embolization were visualized (Figure 4). The serum a-fetoprotein level remained within the normal range (6 ng/mL).

DISCUSSION

Liver RF ablation is an effective minimally invasive therapy used to treat primary and secondary hepatic car-

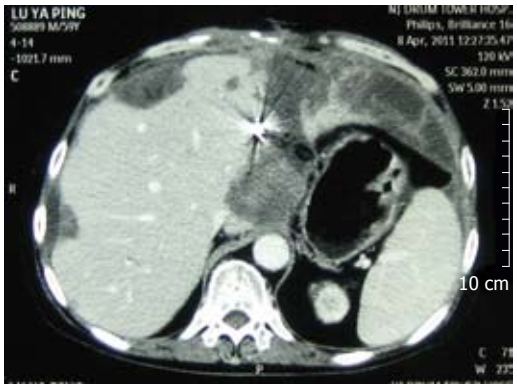

Figure 4 Computed tomography scan acquired 1 mo later. The image revealed that the pseudoaneurysm had disappeared. No tumor enhancement was apparent at the ablated site. The coils used for embolization were visualized.

cinoma[4-6]. In this case, the enhanced imaging of tumors in CT scan was not obvious, suggesting a poor arterial supply. Therefore, we chose RF ablation as the primary treatment rather than transarterial chemo-embolisation. However, the results described in this case report emphasize that hepatic artery rupture may occur after liver RF ablation especially lesions located in caudate lobe and near the hepatic porta.

Hepatic artery rupture is usually associated with intrahepatic abcess, cholangiolithiasis and penetrating trauma[7,8]. Hepatic arterial pseudoaneurysm develops after hepatic artery rupture. Hepatic artery pseudoaneurysms caused by RF ablation were reported previously[9,10]. Hepatic artery pseuoaneurysm arises from the accumulation of blood contained in an aneurismal sac compressed by the liver parenchyma. Rupture of the hepatic artery and subsequent hemorrhage is the cause of death in most patients[9,10]. This is our first experience of hepatic artery rupture in 840 liver RF sessions (0.12%, 1/840) performed during April 2000-Mar 2011. Direct puncture of the hepatic artery by the RF electrode may cause hepatic artery rupture. The thermal injury caused by RF energy may also cause hepatic artery necrosis. The possibility that delayed hemorrhage may develop after liver RF ablation must be kept in mind[9].

An intra-operative ultrasound study and hepatic angiography were useful in detecting the hepatic artery pseudoaneurysm[11,12]. Hepatic angiography is considered the gold standard for diagnosis of hepatic artery injury[13]. We performed emergency hepatic arterial embolization using coils. Hepatic arterial embolization is now the first choice treatment for hemorrhagic pseudoaneurysms because it entails less morbidity and mortality than occur after surgical intervention[14,15]. Experience of hepatic vein pseudoaneurysm caused by RF ablation and managed with stent-graft placement was reported recently[16]. Embolization is reportedly successful in 75% of cases, with a bleeding rate of about 20%.

Laparotomy is an important procedure to control massive bleeding in such an emergent situation. Otherwise there is no opportunity to carry out coil embolization. Due to the severe adhesion of hepatic porta, it is very difficult to dissect the left hepatic artery. Packing with ribbon gauze for hemostasis is a valuable and safe choice but has only a temporary effect. Coil embolization was identified to be the critical treatment for bleeding caused by hepatic pseudoaneurysm. So packing with ribbon gauze and embolizing the hepatic pseudoaneurysm sequentially through angiography is proven to be the ideal strategy of treating such potentially fatal bleeding.

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San Francisco, CA, United States

January 20-21, 2012
AGA Clinical Congress of Gastroenterology and Hepatology: Practice, Evidence and Quality in 2012
Miami, FL, United States

January 27-28, 2012
28th Annual Meeting of the German Association for the Study of the Liver
Hamburg, Germany

January 30-31, 2012
5th International Conference on the Management of Patients with Viral Hepatitis
Paris, France

February 8-10, 2012
Stockholm Liver Week 2012
Stockholm, Sweden

February 16-19, 2012
22nd Conference of the Asian Pacific Association for the Study of the Liver
Taipei, Taiwan, China

March 16 -17, 2012
Hepatitis Single Topic Conference
Atlanta, GA, United States

March 16-17, 2012
ESGE - Workshop on Advanced Endoscopy with Live Demonstrations
Vienna, Austria

March 31-April 1, 2012
27th Annual New Treatments in Chronic Liver Disease
San Diego, CA, United States

April 18-22, 2012
The International Liver Congress by EASL
Barcelona, Spain

April 27-28, 2012
The European Society for Paediatric Gastroenterology, Hepatology and Nutrition
Stockholm, Sweden

May 16-19, 2012
International Liver Transplant Society 18th Annual International Congress 2012
San Francisco, CA, United States

May 19-22, 2012
Digestive Disease Week 2012
San Diego, CA, United States

June 22-23, 2012
EASL Monothematic Conference: Vascular Liver Diseases
Tallin, Estonia

July 1-5, 2012
10th World Congress of the International Hepato-Pancreato-Biliary Association 2012
Paris, France

September 5-8, 2012
International Congress of Pediatric Hepatology, Gastroenterology and Nutrition
Sharm El-Sheikh, Egypt

September 7-9, 2012
Viral Hepatitis Congress 2012
Macclesfield, United Kingdom

September 7-9, 2012
The Viral Hepatitis Congress
Frankfurt, Germany

September 14-16, 2012
The International Liver Cancer Association's 6th Annual Conference
Berlin, Germany

September 20-22, 2012
Prague Hepatology Meeting 2012
Prague, Czech Republic

September 20-22, 2012
1st World Congress on Controversies in the Management of Viral Hepatitis
Prague, Czech Republic

October 18-20, 2012
2nd World Congress on Controversies in the Management of Viral Hepatitis
Berlin, Germany

November 9-13, 2012
AASLD - The Liver Meeting 2012
Boston, MA, United States

November 9-13, 2012
The Liver Meeting - 63rd Annual Meeting and Postgraduate Course of the American Association for the Study of Liver Diseases
Boston, MA, United States

November 14-18, 2012
4th World Congress of Pediatric Gastroenterology, Hepatology and Nutrition
Taipei, Taiwan, China

December 26-28, 2012
International Conference on Gastroenterology, Hepatology and Nutrition
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GENERAL INFORMATION

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Key words

Please list 5-10 key words, selected mainly from *Index Medicus*, which reflect the content of the study.

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Acknowledgments

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Chinese journal article (list all authors and include the PMID where applicable)

2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

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

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

No volume or issue

9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

Statistical data

Write as mean ± SD or mean ± SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

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Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.
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